PERIPHERAL BLOOD STEM CELLS IN HAEMATOLOGICAL MALIGNANCIES

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A thesis submitted for the degree of Doctor of Medicine to the University of Edinburgh, 1991.

CONTENTS

CONTENTS

TABLES

CHAPTER 2

CHAPTER 3

CHAPTER 4

FIGURES

CHAPTER 1

CHAPTER 2

DECLARATION

I declare that this written thesis is my own work, planned, coordinated and performed by myself or under my supervision, unless specifically stated in the acknowledgements.

Jenny I. O. Craig

LIST OF ABBREVIATIONS

ACD-A	Anticoagulant citrate dextrose - solution A
ALL	Acute lymphoblastic leukaemia
AML	Acute myeloid leukaemia
BMT	Bone marrow transplantation
CE	Combined esterase
CFU-C	Colony forming unit - culture
CFU-GM	Colony forming unit - granulocyte monocyte
CFU-mix	Colony forming unit - granulocyte erythroid monocyte megakaryocyte (pluripotential CFU)
CFU-S	Colony forming unit - spleen
CML	Chronic myeloid leukaemia
DFS	Disease free survival
DNA	Deoxyribonucleic acid
DMSO	Dimethyl sulphoxide
DW	Distilled water
EFS	Event free survival
FAB	French American British cooperative group
FCS	Foetal calf serum
G-CSF	Granulocyte colony stimulating factor
GM-CSF	Granulocyte macrophage colony stimulating factor
GVHD	Graft versus host disease
HBSS	Hank's buffered saline solution
HD	Hodgkin's disease
HLA	Histocompatability locus antigen

IgH	Immunoglobulin heavy chain
IgHJ	Immunoglobulin heavy chain joining region
IMDM	Iscove's modified Dulbeccos medium
LCM	Lymphocyte conditioned medium
LFS	Leukaemia free survival
MGG	May Grunwald Giemsa
MIC	Morphologic immunologic & cytogenetic
MNC	Mononuclear cells
MRC	Medical research council
NHL	Non Hodgkin's lymphoma
PBSC	Peripheral blood stem cells
PBSCH	Peripheral blood stem cell harvest
PBSCT	Peripheral blood stem cell tranplant
PCR	Polymerase chain reaction
PHA	Phytohaemagluttinin
Ph'	Philadelphia chromosome
SEM	Standard error of the mean
TCR	T cell receptor
WCC	White cell count

ACKNOWLEDGEMENTS

The diagnosis and management of patients with haematological malignancies depends upon a team of dedicated professionals. I am most grateful to Dr A C Parker, consultant haematologist, who leads this team and whose vision and encouragement enabled this research to be undertaken.

I am indebted to all the patients involved who willingly participated and encouraged me in this work. To my medical and nursing colleagues in the Royal Infirmary of Edinburgh and to Dr C J McCallum, consultant haematologist in Fife I extend my gratitude for help in obtaining samples. The PBSCH were performed in the cell separator unit of the South East Scotland Blood Transfusion Service under the care of Dr J Gillon and by Sister A Stewart and her expert nursing colleagues. My special thanks must go to Staff Nurse Moira Grant who assisted with PBSCH and specific care of the patients in the study. The MLSO staff in the department of Haematology, RIE processed and cryopreserved the PBSCH and gave technical support whenever help was needed.

The mainstay of support came from my colleagues in the Kay Kendall research laboratory in haematology, RIE. Susan Smith, Kenneth Langlands and Anne Anderson comprised a team of workers who gave unending assistance to enable the studies to come to fruition. To Dr Robert Anthony, senior scientist, I am forever indebted for

teaching me the ways of science, guiding me through the peaks and many troughs of research and ultimately delivering me safely back to clinical medicine.
I acknowledge the University of Edinburgh for awarding me a faculty fellowship to carry out this research and the support of the Kay Kendall leukaemia trust.
Finally, to my husband Mark and son Fraser, who managed to arrive in this world in the midst of it all, I dedicate this thesis.

ABSTRACT

Autologous BMT is used successfully as intensive therapy for patients with haematological malignancies and other solid tumours. Peripheral blood contains haematopoietic progenitors whose levels are greatly increased during very early remission after treatment for acute leukaemia or after high dose chemotherapy schedules. These circulating progenitors have been harvested by leucapheresis and used as an alternative to bone marrow as salvage after myelo-ablative treatments. PBSC appear to have several advantages over bone marrow including rapid haematopoietic reconstitution after transplantation, the ability to collect PBSC without a general anaesthetic and in the presence of bone marrow abnormalities and the possibilty of minimal tumour contamination.

My aims were 1) to establish the response of PBSC, measured by the CFU-GM assay, to standard chemotherapy regimes with particular reference to patients with lymphoma, 2) to assess the ability to harvest these progenitors by leukapheresis when numbers were raised and 3) to determine tumour infiltration in PBSC harvests using gene rearrangement studies.

Circulating CFU-GM were studied sequentially in 64 patients (40 female, 24 male), 24 NHL, 22 HD, 9 AML, 5 ALL and 4 myeloma. Three patients with NHL receiving

ALL type therapy showed a mean increase in CFU-GM 15.6 fold over normal values occurring on day 21. Eighteen received CHOP based regimes and demonstrated a 6.2 fold rise between days 15-28. PEEC increased progenitors in 2 patients 43 fold however CVP and VAD only raised CFU-GM by 2.9 and 2.4 fold respectively. In those patients with HD a 14.5 fold increase in PBSC was induced in the 4 patients treated with HOPE. ABVD and MOPP raised peak values to 2.8 and 3.5 times normal and ChlVPP produced a 2.9 fold increase. In the lymphoma group as a whole, standard chemotherapy failed to mobilise PBSC in 15% of patients. Significantly lower peak CFU-GM were found in patients who had received previous chemotherapy or who had marrow involvement with disease.

Four patients with ALL were treated with UKALLX protocol. Induction therapy induced a 5.3 fold rise in CFU-GM occurring on day 21, however intensification produced considerably greater increases to 35 times normal. High dose Ara-C in 1 patient increased PBSC 16.8 fold. Of the 9 with AML 5 received DAT resulting in a mean CFU-GM peak of 6336/ml (38 fold rise). The four remaining patients treated with ADE increased PBSC 12.5 fold. VAD for treatment of myeloma increased PBSC only 1.1 fold. Standard chemotherapy failed to mobilise PBSC in 15% of patients with acute leukaemia and in 75% of those with myeloma.

Having established the timing of PBSC increases, 74 stem cell harvests from 25 patients (8 NHL, 8 HD, 5 AML, 3

ALL, 1 myeloma) were performed using a Fenwall CS3000 cell separator. In patients with lymphoma the mean yield was 0.99 x 10^8MNC/kg per procedure with 2.93 x 10^4CFU-GM/kg. In patients with acute leukaemia 1.2 x 10^8MNC/kg with 8.6 x 10^4CFU-GM/kg per procedure were harvested. The cells were cryopreserved.

Tumour contamination of PBSC harvests was investigated by gene rearrangement studies with probes to IgHJ and TCR β and δ chains. Tumours from 22 patients (3 AML, 4 ALL, 7 NHL, 7 HD and 1 myeloma) and from 60 PBSC harvests were analysed. Rearranged bands were detected in tumour from 10 patients but in none of the PBSC harvests.

In conclusion, standard chemotherapy for lymphoma and leukaemia induced a rise in PBSC in the majority of patients. These progenitors were harvested by leukapheresis in sufficient numbers to allow engraftment. Using gene rearrangement studies, the harvests appeared free of tumour contamination.

The abbreviations for the chemotherapeutic regimes are explained in tables 2.II, 2.III and 2.IV.

CHAPTER 1

INTRODUCTION

Autologous BMT is increasingly used in the treatment of haematological malignancies and solid tumours. The dose of many chemotherapeutic agents and of total body radiotherapy that can be given to treat a large number of tumours is limited by myelosuppression. For sensitive tumours which exhibit a dose-response curve, the probability of eradicating the disease is increased if more intensive treatment is given. Transplantation of stem cells, predominantly from the bone marrow, after high dose therapy is capable of restoring marrow function and thus overcoming the dose limiting effect of myelotoxicity.

1.1. Haematopoietic stem cells

Haematopoietic stem cells have the capacity for self renewal, proliferation and production of progenitors committed by differentiation to the various blood cell lines and therefore are able to permanently support haematopoiesis. The rarity of the most primitive stem cell has led to problems of identification so that studies have required the development of functional assays. Initially the spleen colony assay was devised whereby marrow cells injected into irradiated mice led to the formation of nodules in the spleen. These nodules are

derived from single cells called CFU-S and were shown to consist of differentiating cells of several haematopoietic lineages (Till and McCulloch, 1968). CFU-S are capable of self renewal and differentiation, demonstrated by re-injecting nodules into irradiated mice which subsequently developed splenic colonies. However, CFU-S proved to be heterogeneous in their ability to generate cells capable of long term haematopoiesis and, as was shown recently, many are distinct from the stem cells responsible for its long term maintenance (Jones et al, 1990).

The development of in vitro semi-solid culture methods for quantitation of haematopoietic progenitors has allowed the detection of colonies derived from the committed stem cell which generates the granulocyte/monocyte lineage (CFU-GM or CFU-C) (Bradley and Metcalf, 1966). These are not the equivalent to the CFU-S in mice, but represent an intermediate population between pluripotent stem cells and morphologically recognisable myeloid cells (Wu et al, 1968). The number of CFU-GM was shown to correlate with complete restoration of haematopoiesis after bone marrow transplantation in dogs (Abrams et al, 1981), but in man the results were more variable. CFU-GM correlated with neutrophil recovery but to a lesser degree with platelet recovery (Spitzer et al, 1980). At present CFU-GM is the most generally used predictor of haematopoietic reconstitutive capacity.

Like CFU-S, however, CFU-GM is heterogeneous in its

composition. Monoclonal antibodies against lymphomonocytic and granulocytic surface antigens show 2 types of progenitor cells at different stages of maturation and which are present in different proportions in blood and bone marrow. CFU-GM in peripheral blood are composed almost entirely of a more immature progenitor which differentiates into a second form of CFU-GM found predominantly in the marrow (Ferrero et al, 1983).

Attempts to further identify stem cells in man by methods other than by cell culture, have employed flow cytometry. Flow cytometric analysis has demonstrated the number of cells expressing the antigen CD34 (CD34+ cells) present in the peripheral blood after stem cell mobilisation correlated with CFU-GM from culture experiments (Siena et al, 1991; Serke et al, 1991). Subpopulations of CD34+ cells have been demonstrated and the best predictor early haematopoietic recovery after PBSCT found to be the number of cells that co-expressed the antigen CD33 (CD34+/CD33+) (Siena et al, 1991). Cells with phenotype CD34+/CD33- may be responsible for the later sustained engraftment phase and contain the most primitive haematopoietic cells. This hypothesis was supported by recent evidence that cells from marrow that were CD34+ but negative for T, B and myeloid cell antigens have generated multiple colony forming cell progeny (Andrews et al, 1990). Thus the positive identification of the true human stem cell is coming closer.

1.2. The role of transplantation in haematological malignancies

1.2.1. Lymphoma

In patients with NHL, more than 50% will relapse after conventional chemotherapy and less than 10% of such patients are cured (Cabanillas et al, 1982; Velasquez et al, 1988). Dose escalation, as achieved by autologous transplantation, produced a complete response in 14% of patients with aggressive NHL resistant to standard treatment after a follow up of 3 years (Philip et al, 1987). There are no direct prospective comparisons in NHL between allogeneic and autologous BMT but comparing the few reports of allogeneic with those of autologous BMT there is no advantage in survival or frequency of relapse in either procedure (Armitage, 1989). In a review of over 500 patients with intermediate and high grade NHL, the best results of autologous transplantation were in those with minimal tumour, early in the course of disease (complete response or sensitive relapse) where 50% were alive and well with follow up from 1 to 75 months (Armitage 1989). The optimal timing of BMT in NHL is not yet established, but if poor prognostic factors such as a slow response to treatment (Armitage et al, 1986) can be identified it may have a role in first remission.

In HD the situation is more complex with two thirds of patients cured by local radiotherapy or chemotherapy (Selby et al, 1987). A group with a poor prognosis can be identified and includes those who do not completely

respond to initial treatment or who do not respond to second line therapy after relapse. BMT has been used mainly for advanced cases of HD and even in these cases, a 50% complete response rate was seen (Gribben et al, 1989; Armitage, 1989).

1.2.2. Acute leukaemia

For those patients with AML conventional chemotherapy has improved over the last 20 years and now produces remission in 76% of adults below 50 years of age (Rees et al, 1986), with an overall LFS of 25 - 40% at 3 years (Butturini and Gale, 1989). Allogeneic BMT is an effective treatment with 48% LFS at 5 years for those transplanted in first remission (Advisory Committee of the International Bone Marrow Transplant Registry, 1989). The role of autologous BMT in AML is less well defined and is under prospective evaluation in the MRC AML X trial. Other non-controlled studies have suggested a 36% LFS at 7 years with autologous transplantation and after in vitro chemotherapeutic marrow purging the European BMT group have reported LFS of 60% at 5 years (Gorin et al, 1989).

Modern chemotherapy regimes can cure approximately one third of adults with ALL (Hoelzer and Gale, 1987), whereas allogeneic BMT in first remission produces a LFS of 40%. In second remission the LFS after BMT is 20% compared with 5% after reinduction chemotherapy (Butturini and Gale, 1989). Therefore BMT is appropriate in first remission ALL only for those with poor

prognostic features such as a high presenting white cell count or a slow response to induction chemotherapy (Hoelzer et al, 1988), and in second remission for those with standard risk disease. As the numbers of adults with ALL is relatively small, the role of autologous transplantation has still to be established and the use of marrow purging with chemotherapy or specific monoclonal antibodies evaluated. The European BMT group report a LFS of 41% at 3 years in poor prognosis ALL after autologous transplantation in first complete remission, but in only 10% during second remission (Gorin et al, 1989).

1.3. Complications of BMT

BMT is a major procedure with an overall mortality of 20-50% (Champlin and Gale, 1984). One of the main limiting factors in allogeneic BMT is the availability of a suitable donor. A patient has a 1 in 4 chance of having an HLA identical sibling, but the size of western families means that such a donor is available for about 1/3 of patients (Black, 1982). Even with an appropriate donor, GVHD occurs with a mortality of 30% in moderate to severe cases (Storb et al, 1983). The use of autologous marrow overcomes the problem of stem cell rescue and removes the risk of GVHD, but the complications of marrow hypoplasia such as bleeding and infection, which is responsible for 10-20% of mortality in some reports, remains. The disadvantages of autologous BMT lie with the potential for disease relapse. Tumour cells may be rein-

fused with the marrow, but quantification of this risk is impossible in view of the relatively insensitive assays for tumour contamination in either the patient or graft. The loss of the benefit of the graft versus leukaemia effect of allogeneic marrow (Sosman and Sondel, 1991) may make relapse more likely.

1.4. Peripheral Blood Stem Cells

The peripheral blood represents an alternative source of stem cells to bone marrow. Progenitor cells are present in the mononuclear cell fraction of peripheral blood in both animals and man, and could be used to potentially increase the use of autologous transplantation.

1.4.1. PBSC in animals

That buffy coat cells could protect irradiated parabiotic mice from bone marrow failure was first documented in 1951 (Brecher and Cronkite, 1951). Using transplantation experiments it was shown that in mice given blood-derived white cell transplants, granulocytes and erythroblasts were of donor origin and chimeras were established for several months (Goodman and Hodgson, 1962). At an early stage, the importance of the dose of transfused white cells was shown in dogs (Cavins et al, 1964), when 10 times more peripheral blood was required to give haematological recovery compared with that of bone marrow.

When the properties of circulating haematopoietic stem cells were compared to the known marrow derived stem

cells, they were found to differ by having a lower radiosensitivity, more cells in S phase and a higher seeding efficiency (Gidali et al, 1974). Micklem and his colleagues found the self-renewing capacity of peripheral blood CFU-S was less than those in the marrow (Micklem et al, 1975) and it was considered that they represented a more committed fraction of the stem cell population.

Development of the CFU-C assay, in which murine haematopoietic cells could form granulocyte and monocyte colonies in semisolid medium supplemented with growth factors (Bradley and Metcalf, 1966), enhanced the study of stem cells. CFU-C were found to be a good predictor of engraftment of peripheral MNC (Northdurft et al, 1977) and the importance of dose was becoming apparent. In dog experiments a nucleated cell count of more than 3 x 10^8/kg and CFU-C of 1.9 - 9.9 x 10^4/kg resulted in sustained engraftment for up to 3 years.

Harvesting of progenitors was facilitated in dogs by treatment with cyclophosphamide to increase the circulating pool of CFU-C (Abrams et al, 1981). This not only increased the CFU-C 11 fold but also increased haematopoietic recovery 12.5 fold after transplantation.

These encouraging results in animal experiments led on to work in the human situation.

1.4.2. PBSC in man

Cells capable of forming colonies are present in the

peripheral blood of man. This was first demonstrated by Chervenick and Boggs in 1971 who observed colonies containing granulocytes and monocytes in the feeder layers of marrow cultures made from blood leukocytes (Chervenick and Boggs, 1971). At the same time Kurnicke cultured peripheral blood cells from patients with infectious mononucleosis and other viral illnesses with an increased number of circulating large lymphocytes and found a greater number of granulocytic colonies than in his normal population (Kurnick and Robinson, 1971). By a series of depletion and separation experiments Barr showed the stem cell activity which produced granulocyte and erythroid colonies was found in a population of large lymphocytes (Barr et al, 1975). Erythroid and granulocytic colonies in peripheral blood, bone marrow and cord blood were also observed (Fausner and Mesner, 1978).

1.5. Stem Cell Mobilisation

Several factors were shown to mobilise PBSC. CFU-C were increased four fold by exercise, two fold by ACTH induced stress (Barrett et al, 1978) and hydrocortisone (Morra et al, 1981), and four fold by endotoxin (Cline and Golde, 1977). In certain disease states, particularly myeloproliferative disorders such as myelofibrosis and CML greatly increased numbers of circulating progenitors were found (Chervenick, 1973;

Hibben et al, 1984; Partanen, et al, 1982). The effects of chemotherapy were first demonstrated by Richman in 1976 who reported up to a twenty fold rise in circulating CFU-C after intermittent chemotherapy for solid tumours. She postulated it would be possible in man to obtain a dose of stem cells with a small number of leukaphereses, equivalent to a bone marrow harvest at the time of CFU-C expansion (Richman et al, 1976).

The response of circulating stem cells in AML measured by the CFU-GM assay was initially reported by To (To et al, 1984). He found, in those patients who entered remission, a twenty five fold increase in CFU-GM over normal subjects occurred, which coincided with the platelet count recovery. A mean of 11 x 10^4 CFU-GM/kg were harvested by leucapheresis and cryopreserved without loss of viability. Similar increases in PBSC were reported predominantly in AML but also in a small number of patients with ALL (Bernard et al, 1985; Reid et al, 1989). Recently the administration of very high doses of chemotherapy specifically to mobilise PBSC, was reported with increased progenitors found in 69% of patients. However, this was associated with significant morbidity and even mortality (To et al, 1990).

During very early remission in AML, the ratio of the more primitive pluripotent cell, CFU-mix, to CFU-GM was reported to be increased (Geissler et al, 1986). However, this was not confirmed by others (Juttner et al, 1986;

Reid et al, 1989) and thus the predictive capacity of CFU-mix for engraftment remains unclear. This also applies to bone marrow transplantation where in vitro treatment of marrow with 4-hydroperoxycyclophosphamide destroyed CFU-mix without loss of transplantation potential (Rowley et al, 1985).

1.6. PBSC Autografting

1.6.1. Early studies

Transplantation experiments in animal models showed it was feasible to obtain adequate numbers of stem cells from the peripheral blood by leukapheresis to allow reconstitution of haematopoiesis (Debelak-Fehir et al, 1975; Cavins et al, 1964; Northdurft et al, 1977), particularly if the stem cell pool was expanded, for example, by dextran sulphate or cyclophosphamide (Gerhartz et al, 1985; Abrams et al, 1981). In man it became apparent that blood contained cells capable of establishing haematopoiesis when some neutropenic patients treated with buffy coats from patients with CML, transiently developed Ph' positive haematopoiesis (Freireich et al, 1964). However, the first use of PBSC for autografting in man was in CML where cryopreserved buffy coat cells from patients with chronic phase disease were used to restore haematopoiesis after ablative therapy for accelerated phase or blast crises. (Goldman et al, 1979). Marrow function was rapidly restored but

relapse inevitably occurred. Normal stem cells from a chemotherapeutically induced Ph' negative phase of CML were also transplanted by Korbling with complete haematopoietic Ph' negative reconstitution in the short term (Korbling et al, 1981). Ph' negative haematopoiesis was also restored by transplantation of CML buffy coat cells grown in liquid culture in vitro to allow preferential expansion of the normal stem cell over those belonging to the malignant clone (Marcus and Goldman, 1986).

Early attempts at grafting using PBSC derived from identical twins were unsuccessful, probably due to low CFU-GM dose and long periods of reinfusion. The first was performed for marrow aplasia in a patient with paroxysmal nocturnal haemaglobinuria who received 3.4×10^4 CFU-GM/kg over a 2 week period with no conditoning but failed to engraft (Hershko et al, 1979). The second patient had a Ewing's sarcoma and after conditioning, received 0.9×10^4CFU-GM/kg obtained by 8 leukaphereses from his sibling The cells were reinfused over several days but did not result in an acceleration of granulocyte, monocyte or platelet recovery (Abrams et al, 1980).

1.6.2. PBSCT in leukaemia

The reconstitutive ability of PBSC in leukaemia has been established but the therapeutic effects of PBSCT are not yet clear. Initial case reports on the use of PBSCT in 2 patients with relapsed AML showed early engraftment and

early relapse (Juttner et al, 1985). A further patient, however, remained in remission for at least 7 months with sustained haematopoiesis (To et al, 1987a). Various other case studies differed, suggesting that in first and second remission, response could be either sustained or that early relapse may occur (Reiffers et al, 1988; Bell et al, 1988). In a relatively large study of 26 patients transplanted in first complete remission, Reiffers estimated a DFS at 3 years of 33%, which is similar to that of autologous bone marrow transplantation. These 2 procedures are being prospectively compared (Reiffers et al, 1990).

Even fewer PBSCT for ALL have been reported, but 2 case studies found good engraftment with remission continuing up to 6 months (Bell et al, 1987b; Tilly et al, 1986).

1.6.3. PBSCT in lymphoma

PBSC may also be of benefit in the treatment of patients with lymphoma. A 25% 2 year DFS was reported after PBSC transplant for malignant lymphoma in dogs, this was similar to results obtained after BMT (Appelbaum et al, 1986). The first human with lymphoma to undergo PBSCT was a patient with Burkitt's lymphoma who was treated with high dose chemoradiotherapy followed by reinfusion of PBSC. The patient remained well during the 7 months follow up period (Korbling, et al, 1986).

Kessinger has reported the results of PBSC transplantation in the largest series of patients with

lymphoma. Sixteen patients with NHL and 24 with HD were studied. All had refractory disease and had received much previous therapy. Many had marrow involvement or had received pelvic radiotherapy. The clinical response compared well with autologous BMT with a 2 year EFS of 49% for those with NHL and an 18 month EFS of 15% in HD (Kessinger et al, 1989a).

1.6.4. PBSCT in Myeloma

In one study, PBSCT in myeloma produced considerable tumour reduction in 87% and a complete response in 25% of 8 patients with stage III aggressive disease (Fermand et al, 1989). When used as early intensive treatment a similar response was found, with 25% remaining in CR at 5 and 14 months (Bell et al, 1990). The long term results and role of this mode of therapy still have to be established.

Stem cells in the peripheral blood can be harvested and successfully establish haematopoiesis. In the small numbers of patients studied, the results of PBSC transplantation compare favourably to those found after BMT and thus this form of treatment deserves further study.

1.7. Advantages of PBSC

1.7.1. Collection of PBSC

The advantages that PBSC have over bone marrow are that they can be harvested without the prerequisite of a healthy pelvic bone marrow and without the need for a general anaesthetic, therefore if a patient has marrow fibrosis as a result of previous radiotherapy or marrow infiltration, this therapeutic option is still available.

As the stem cell activity is found in the lymphocyte fraction of peripheral blood, leukapheresis is used to harvest progenitors. Many cell separators employing continuous and discontinuous flow have been used successfully such as the Aminco centrifuge (Korbling et al, 1980), the Hemonetics Model V50 (Schouten et al, 1990), the Cobe Spectra (Hillyer et al, 1990) and the Fenwall CS3000 (Lasky et al, 1982). The more recent protocols and use of separators with computer control have increased efficiency and ease of the procedure (To et al, 1989). After collection, harvests have been cryopreserved with or without further manipulation, mainly using 10% by volume of DMSO as cryoprotectant and freezing using a controlled rate freezer before being stored in liquid nitrogen (Korbling et al, 1988). The products of several leukapheresis procedures are generally reinfused at transplant, therefore purification of stem cells to avoid side effects from the infusion of large amounts of DMSO and contaminating red cells (Kessinger et al, 1990) and to eliminate post-thaw granulocyte clumping, may be performed. Density gradient

separation of the product manually or by machine (Hillyer et al, 1990; Law et al, 1988) will inevitably result in the loss of some cells but Douay has suggested that without Ficoll separation there is a significant loss of CFU-GM after cryopreservation (Douay et al, 1989).

1.7.2. Rapid haematopoietic reconstitution

The observation that haematopoietic reconstitution is rapid after PBSCT offers the advantage of a shorter period of neutropenia, the potential for a lower infection rate and subsequent mortality. Rapid reconstitution is seen in those patients transplanted with PBSC collected after mobilisation by chemotherapy (Korbling and Martin, 1988) but recovery rates similar to autologous marrow transplantation occur when progenitors are collected during steady state haematopoiesis (Kessinger et al, 1988).

1.7.3. Lower tumour contamination

A further potential advantage of PBSC is the possibility of lower levels of tumour contamination than in bone marrow. If this were the case it may prevent some relapses after transplantation. It is well recognised that relapse is also due to residual disease in the recipient therefore conditioning regimes also need to be improved. One report suggesting that PBSC may have less tumour contamination was that of To who employed morphological and cytogenetic techniques to examine a

harvest from a patient who had AML with the blast cells containing Auer rods and a specific chromosomal translocation. No leukaemic blasts were found in the PBSC harvest using these techniques the sensitivity of which was 2% (To et al, 1987b). Bell compared IgHJ gene rearrangements in PBSCH with bone marrow from patients after treatment for myeloma and found the incidence of tumour contamination was less (Bell et al, 1990).

1.8. Disadvantages of PBSC

1.8.1. CFU-GM dose

Juttner reported the initial experience in AML involving 2 patients with relapsed disease who received 29 and 23 x 10^4 CFU-GM/kg and both demonstrated initial haematopoietic reconstitution. However, one relapsed and died at day 11 and the other showed a secondary fall in blood counts which remained persistently low after day 16 until the patient relapsed (Juttner et al, 1985). When higher doses of CFU-GM (230 x 10^4/kg) were given to another patient, normal blood counts were reached by day 14 and did not fall thereafter. After further experience this group concluded that rapid and complete engraftment in AML required doses of CFU-GM greater than 63 x 10^4/kg (To et al, 1986). Other workers confirmed rapid and sustained recovery of counts although CFU-GM doses varied, eg Henon reported neutrophils >0.5 x 10^9/l at 10

days and platelets > 50 x 10^9/l at 18 days in patients receiving 39 x 10^4CFU-GM/kg (Henon et al, 1988).

In non-leukaemic patients the dose of stem cells required for engraftment is also variable, but appears to be less than in patients with AML. The first patient with lymphoma to undergo PBSCT received 21 x 10^4 CFU-GM/kg collected in 7 leukaphereses after chemotherapy and cryopreserved (Korbling and Martin, 1988). He showed rapid haematopoietic reconstitution with neutrophils >0.5 x10^9/l and platelets > 50 x 10^9/l both on day 10. Of 2 patients with NHL who received PBSC collected after chemotherapy, one, transplanted with 60 x 10^4 CFU-GM/kg showed a rapid and complete haematopoietic reconstitution, but the second patient, given a smaller dose (33 x 10^4CFU-GM/kg), died on day 21 with severe hypoplasia (Bell et al, 1987b). Stiff noticed incomplete platelet reconstitution in patients with lung tumours transplanted with a mean of 11.4 ± 9.9 x 10^4 CFU-GM/kg (Stiff et al, 1987), whereas Lasky infused 1.1 - 5.7 x 10^4 CFU-GM/kg to 7 patients with HD and reported sustained engraftment (Lasky et al, 1989). Korbling transplanted 3 patients with lymphoma who received PBSC mobilised after chemotherapy and found rapid and complete recovery when 6.5 x 10^4 CFU-GM/kg were infused, but an incomplete platelet response if the patients received < 2 x 10^4/kg (Korbling and Martin, 1988).

PBSC have also been harvested under steady state conditions and transplanted but reconstitution has been

no faster than with bone marrow (Williams et al, 1990; Phillips et al, 1990). In Kessinger's series of lymphoma patients a mean of 8 PBSC collections during steady state haematopoiesis were performed harvesting 7 x 10^8 MNC/kg with 0.6 x 10^4CFU-GM/kg. Engraftment was similar to that found in bone marrow transplants taking 25 days to increase neutrophils to over 0.5 x 10^9/l (Kessinger et al, 1989).

1.8.2. PBSC mobilisation and prediction of flux

The nature of the CFU-GM assay requiring a 2 week incubation period makes it impossible to give a rapid result and thus provide a clinically useful indication as to when levels of circulating CFU-GM are high and suitable for harvesting. Indirect measurements of the appearance of PBSC such as the synchronous recovery of monocytes and platelets in patients with AML (Reid et al, 1989), or a rapid rise in white cell count (Cantin et al, 1989) have been used. In order to specifically mobilise stem cells, high dose chemotherapy such as cyclophosphamide (4g/m^2) were given. This mobilised high numbers of CFU-GM at predictable times in 69% of patients but with considerable morbidity and one death (To et al, 1990). The recent demonstration of immunophenotypic methods for CD34 estimations which correlate with stem cells may overcome this to some extent.

1.8.3. Quality of PBSC

The quality of PBSC in comparison to bone marrow progenitors and their ability to maintain long-term haematopoiesis has also been questioned predominantly based on results from mouse experiments (Micklem et al, 1975). Successful long term reconstitution has, however. been demonstrated in dogs (Northdurft et al, 1977) and in several studies in man (Reiffers et al, 1990; Kessinger et al, 1989).

In this study, variations in the levels of PBSC were examined, using the CFU-GM assay, in patients with haematological malignancies to determine the effects of standard chemotherapy regimes on their levels at various time intervals. Having established the timing of PBSC flux after standard chemotherapy, the feasability of collecting progenitors was assessed by performing PBSC harvests using a CS3000 cell separator. Finally, the degree of tumour contamination in the harvests was investigated by gene rearrangement studies.

1.9. Tumour contamination of PBSCH

The presence of tumour cells in PBSCH may be an important source of disease from which relapse could occur. Differentiating tumour from stem cells and other mononuclear cells in the harvest can be attempted by various methods such as light microscopy, cytogenetic studies, and immunophenotypic analysis. All these

systems depend upon the recognition of a tumour marker.

1.9.1. Immunological genes

DNA analysis of Ig and TCR genes has proved a useful tool for the detection of residual disease in some haematological malignancies. Antibody molecules are proteins made from two heavy chains and two light chains. Antigen binding sites on antibody molecules consist of a cleft composed of two side walls. Each wall is made of three polypeptide loops from either a heavy chain variable region or a light chain variable region. Diversity of the shape of the cleft results from differences in the amino acid sequence of these heavy and light chain variable regions. Amino acid variability is achieved by recombination of encoding DNA sequences. (Tonegawa, 1983). During early B cell development segments of DNA from IgH variable, diversity and joining regions, situated on chromosome 14, are brought together by DNA recombination and this rearranged sequence is transcribed into RNA (fig 1.1). The light chain gene V and J segments from chromosome 22 recombine in a similar manner. The vast numbers of different combinations generate the large repertoire of antibody molecules (Tonegawa, 1983). Further variability is introduced by the imprecise nature of the V-D-J fusion and the non specific addition of nucleotides at recombination. As the DNA nucleotide sequence must be aligned correctly for translation of an in-frame amino acid sequence, during

recombination stop codons tend to be generated resulting in a non productive rearrangement. If this happens, the process is repeated using the second allele in order to produce a functional antibody.

The T cell receptor protein consists of α/β chains in the vast majority of mature T cells, or in a minority, of Γ/δ chain combinations found predominantly on CD4-/CD8- cells (Knowles, 1989). The V, D and J segments which encode the Variable TCR regions, recombine in a similar fashion to IgH V-D-J segment rearrangements.

The repertoire of B and T cells consists of very large numbers of immune lymphocytes, each with different V-D-J rearrangements of either IgH and light chains or TCR α/β or Γ/δ chains. These gene sequence rearrangements may be analysed by restriction enzyme cleavage, agarose gel electrophoresis to separate fragments of DNA by size, Southern blotting and hybridisation with a DNA probe complementary to part of the rearranged sequence. In the normal immune cell repertoire, gene rearrangement studies identify one or two bands due to unrearranged germline alleles and a smear generated by the large number of cells with different V-D-J rearrangements. However, in the case of neoplastic transformation, there is clonal expansion of a single malignant lymphocyte containing a specific V-D-J segment which can be detected by gene rearrangement studies as a single band (Rabbitts, 1986).

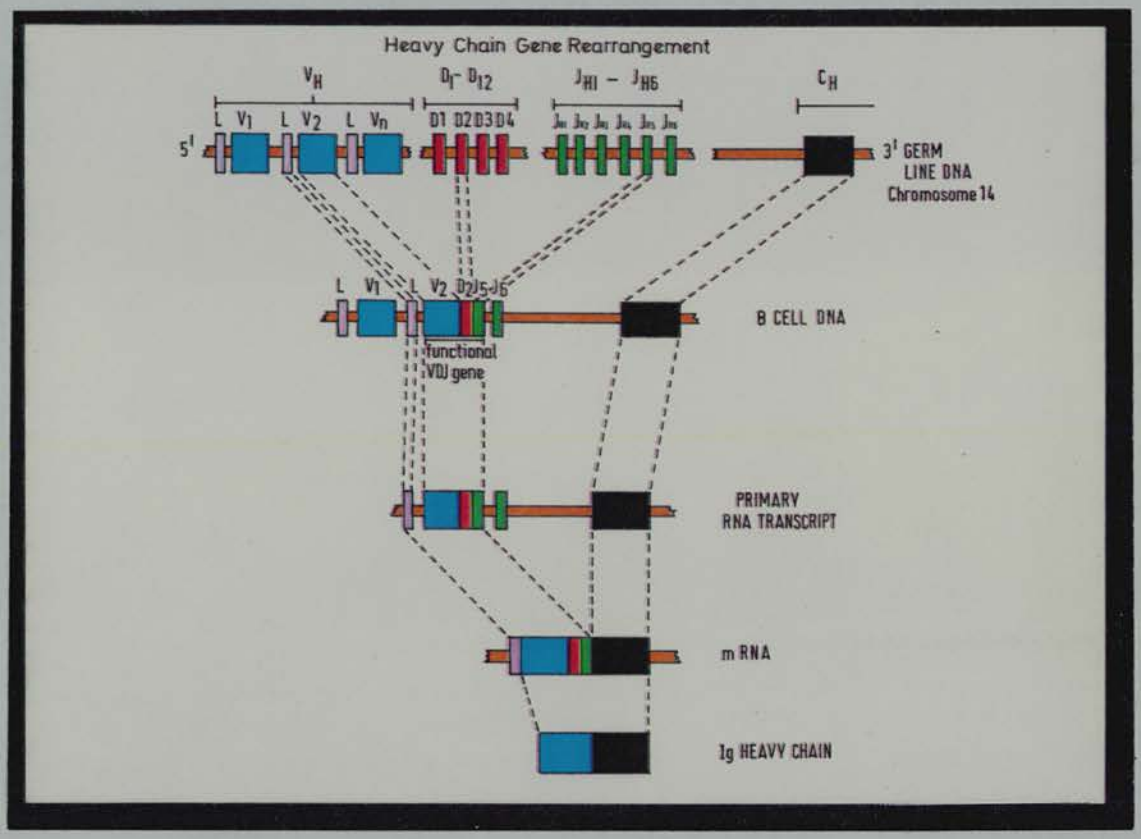

Figure 1.1

Immunoglobulin heavy chain gene recombination. During B cell development, antibody diversity is created by the recombination of any one variable (V) segment with any one diversity (D) segment and any one joining (J) segment of DNA to produce a functional V-D-J gene. This is then transcribed into mRNA and translated into IgH protein.

Gene rearrangement studies have proved useful in assigning lineage to lymphoid tumours, but in approximately 15% of patients reported, both TCR and IgH genes are rearranged. (Pellici et al, 1985; Tawa et al, 1985; O'Connor et al, 1987; Seremetis et al, 1987). In NHL, IgHJ gene rearrangements were found in all tumours of B cell origin in 2 reported series (Aisenberg et al, 1987; Williams et al, 1987) and TCRβ gene rearrangements in 96% of T cell NHL (Williams et al, 1987; Griesser et al, 1986). In HD the cell of origin is still controversial and most tumours do not demonstrate gene rearrangements. An average of 9% of 117 patients from 7 reported series showed IgHJ gene rearrangements, 9% TCRβ gene rearrangements and 28% of 7 patients TCR δ gene rearrangements. (Greissler et al, 1986; Brinkler et al, 1987; Sundeen et al, 1987; O'Connor et al, 1987; Henni et al, 1988; Tkachuk et al, 1988; Gledhill et al, 1990). An average of 7% of 204 patients with AML from 5 reported series showed IgHJ and 14% TCR β gene rearrangements (Cheng et al, 1986; Seremitis et al, 1987; Ackland et al, 1987; Foroni et al, 1989; Dyer et al, 1988). TCR δ gene rearrangements have been reported in 32% of 65 patients with AML from 3 studies (Foroni et al, 1989; Dyer et al, 1988; Asou et al, 1989). In those patients with B cell lineage ALL almost all had IgHJ rearranged (Korsameyer et al, 1983) and in 5 reported series of 50 patients with T cell lineage ALL, 92% demonstrated TCRβ rearrangements (Pellici et al, 1985; Waldemann et al,

1985; Knowles et al, 1986; Felix et al, 1987; O'Connor et al, 1987).

Circulating clones of cells detected by gene rearrangement studies have been found in 38% of untreated or relapsed patients with NHL, most commonly in advanced disease and low grade disease (Brada et al, 1987). A similar result was seen by Horning who found 34% positive with low grade NHL and only 8 % in intermediate disease. When patients were clinically free of disease irrespective of grade, DNA analysis of peripheral blood was still positive in 10% (Horning et al, 1990).

Gene rearrangement studies have provided tumour markers in the majority of patients with acute leukaemia and NHL and in some with HD and AML. This approach was used to investigate tumour contamination of peripheral blood stem cell harvests.

1.10. AIMS

The aims of this project were as follows:

To investigate the response of PBSC to standard chemotherapy used in the treatment of haematological malignancies and to determine the pattern of variation in numbers.

To investigate the feasibilty of collection of these progenitor cells after standard chemotherapy.

To assess the contamination of PBSC harvests by malignant cells.

CHAPTER 2

PERIPHERAL BLOOD STEM CELLS

The response of peripheral blood stem cells to standard chemotherapy regimes for the treatment of haematological malignancies was investigated.

2.1. Materials

Ficoll-hypaque 1.077g/ml (Pharmacia, Uppsala, Sweden)
FCS (Northumbria Biological), heat inactivated at 56°C for 30 min to destroy complement
Gentamicin (Sigma, Poole,Dorset, UK.)
HBSS (Northumbria Biological)
IMDM (Gibco/BRL, Paisley, UK)
2 mercaptoethanol (Sigma)
Methylcellulose 4000 cps (Sigma)
Penicillin and streptomycin solution (Northumbria Biological, Cramlington, Northumbria, UK.)
PHA (Wellcome Diagnostics, Dartford, UK)
Preservative free heparin (Leo Laboratories, Princes, Risborough UK)

All plastic-ware was from Costar, Massachusettes

2.2. Subjects and patients

2.2.1. Normals

To establish normal values for PBSC, CFU-GM from 43 healthy volunteers (19 male, 24 female, mean age 30.3 years, range 15 - 57) were assayed throughout the study period.

2.2.2. Patients

Circulating CFU-GM were measured on serial blood samples from 64 patients with haematological malignancies (Table 2.1) undergoing standard chemotherapy. AML was classified according to the FAB criteria (Bennett et al, 1985), ALL according to MIC criterea (First MIC Cooperative Study group, 1986), and NHL according to the Keil classification (Stansfield et al, 1988). Patients with lymphoma were clinically staged according to the Ann Arbor system (Carbone et al, 1971). Bone marrow involvement was assessed by light microscopic examination of marrow aspirate and trephine biopsy.

Of the 24 patients with NHL, 21 had high grade, 3 low grade disease, one stage I, one stage II, 7 stage III and 13 stage IV disease. In those with HD, 3 had stage I, 4 stage II, 4 stage III and 11 stage IV.

The FAB classification of AML gave 4 M1, one M2, one M3, 2 M4 and one M5. The MIC classification of the patients with ALL gave 3 common ALL, one pre-B ALL and one pre-T ALL.

Disease	n	mean age (range y)	M/F	previous therapy
NHL	24	49.1 (17-68)	12M 12F	2
HD	22	31.9 (14-66)	15M 7F	6
AML	9	47.3 (32-70)	6M 3F	0
ALL	5	29 (17-51)	4M 1F	0
Myeloma	4	58.5 (54-62)	3M 1F	3
Total	64	41.6 (14-70)	40M 24F	11

TABLE 2.I Characteristics of patients receiving chemotherapy

The chemotherapy regimes used are described in tables 2.II to 2.V. Eighteen patients with NHL received CHOP-based regimes (hereafter referred to as CHOP), 16 being BCHOP-M as described in table 2.II, the other 2 omitting bleomycin and methotrexate. Cycles were repeated every 21 days, counts permitting. CVP was given every 4 weeks and VAD every 6 weeks.

Patients with HD received ChlVPP, ABVD, MOPP, HOPE and OPEC every 4 weeks.

BCHOP-M bleomycin 7.5mg/m^2 iv, cyclophosphamide 750mg/m^2 iv, adriamycin 40mg/m^2 iv, vincristine 1.4mg/m^2 iv all day 1, prednisolone 40mg po days 1-5, methotrexate 200mg/m^2 iv day 15 with oral folinic acid rescue.

ALL daunorubicin 45mg/m^2iv days 1 & 2, vincristine 1.5mg/m^2 iv days 1,8,15,22 & 29, prednisolone 40mg/m^2 po days 1-28, asparaginase 6000U/m^2 sc 3/week x 9.

PEEC methylprednisolone 250mg iv, vindesine 3mg/m^2 both day 1, etoposide 100mg/m^2 iv day 1, 200mg/m^2 po days 2 & 3, chlorambucil 20mg/m^2 po days 1,2 & 3, methotrexate 200mg/m^2 iv day 15 with oral folinic acid rescue.

CVP cyclophosphamide 1g iv and vincristine 2mg iv day 1, prednisolone 40mg po days 1-5.

VAD vincristine 0.4mg/m^2 and adriamycin 9mg/m^2 iv days 1-4, dexamethasone 40mg daily po days 1-4, 9-12 and 17-21.

TABLE 2.II NHL Chemotherapy regimes

ChlVPP chlorambucil 6mg/m^2 po, procarbazine 100mg/m^2 po and prednisolone 40mg po daily all days 1-14, vinblastine 6mg/m^2 iv days 1 & 8.

ABVD adriamycin 25mg/m^2 iv, bleomycin 10mg/m^2 iv, vinblastine 4mg/m^2 iv and DTIC 375mg/m^2 iv all days 1 & 15.

MOPP procarbazine 100mg/m^2 po, prednisolone 40mg/m^2 po both days 1-14, mustine 6mg/m^2 iv and vincristine 1.4mg/m^2 iv both days 1 & 8.

HOPE adriamycin 40mg/m^2 iv day 1, vincristine 1.4mg/m^2 iv days 1 & 8, prednisolone 100mg po days 1-8, etoposide 200mg/m^2 po days 1-4, bleomycin 10 mg iv days 1 & 8.

OPEC chlorambucil 6mg/m^2 po and prednisolone 40mg po daily days 1-14, etoposide 200mg/m^2 po days 1-5, vincristine 1.4mg/m^2 iv days 1 & 8.

TABLE 2.III HD chemotherapy regimes

UKALL (INDUCTION) daunorubicin 45mg/m^2 iv days 1 & 2, vincristine 1.5mg/m^2 iv days 1,8,15,22 & 29, prednisolone 40mg/m^2 po days 1-28, asparaginase 6000U/m^2 sc 3/week x 9.

UKALL (INTENSIFICATION) prednisolone 40mg/m^2/day po days 1-7 stopping over 1 week, vincristine 1.5mg/m^2 iv day 1, daunorubicin 45mg/m^2 iv days 1 & 2, etoposide 100mg/m^2 iv days 1-5, cytosine arabinoside 100mg/m^2 bd iv days 1-5, thioguanine 80mg/m^2 po days 1-5

MACHO cyclophosphamide 300mg/m^2 bd iv days 1-3, vincristine 1.5mg/m^2 iv days 4 & 11, adriamycin 50mg/m^2 iv day 4, methotrexate 2.5g/m^2 iv day 15 with iv folinic acid rescue, on recovery, ara-C 2g/m^2 bd iv for 3 days followed by methotrexate 2g/m^2 iv 15 days later with iv folinic acid rescue.

DAT 3+10 daunorubicin 50mg/m^2 iv days 1, 3 & 5, cytosine arabinoside 100mg/m^2 iv bd days 1-10, thioguanine 100mg/m^2 po days 1-10. Note DAT 1+5 has 1 day of daunorubicin and 5 days of cytosine and 6TG.

ADE cytosine arabinoside 100mg/m^2 iv bd days 1-10, daunorubicin 50mg/m^2 iv days 1, 3 & 5, etoposide 100mg/m^2 iv days 1-5.

TABLE 2.IV Acute leukaemia chemotherapy regimes

2.3 Methods

Sterile reagents, equipment and technique were employed throughout cell culture work.

2.3.1. CFU-GM assay

PBSC were assessed in a semi solid culture system using the CFU-GM progenitor assay based on a modification of that of Fausner and Messner (1978). Twenty ml of peripheral blood were collected into 300U of preservative free heparin and then diluted 1:1 with HBSS. The light density MNC were isolated by density gradient centrifugation over Ficoll-hypaque (1.077g/ml) at 400g for 30 min according to Boyum, 1964. The MNC were removed, washed twice in HBSS + 2% FCS and resuspended in IMDM + 2% FCS. Cells were plated at 4×10^5/ml in IMDM with 1.44% methylcellulose, 30% FCS, 10% PHA LCM, 250μg/ml gentamicin, 100u/ml penicillin and 100μg/ml streptomycin. Cultures were set up in duplicate in 22.6mm diameter wells in 12 well tissue culture plates, incubated at 37°C in a humidified atmosphere containing 5% CO_2 for 14 days and then colonies >40 cells were counted using an inverted microscope.

2.3.2. Preparation of PHA LCM

From the same donor 20 ml peripheral blood were collected into 300u preservative free heparin and the MNC isolated by density gradient centrifugation over Ficoll-hypaque as

described above. The MNC were washed twice in HBSS + 2% FCS and resuspended in IMDM + 2% FCS to give a final concentration of 1 x 10^6 MNC/ml containing 1% PHA, 10% FCS, 50μM 2 mercaptoethanol and antibiotics (Gentamicin 250μg/ml, penicillin 100u/ml and streptomycin 100μg/ml) in IMDM. This was incubated at 37°C in a humidified atmosphere containing 5%CO_2 for 4 days when it was decanted, centrifuged at 200g for 10 min and the supernatant (PHA-LCM) filter sterilised and frozen at -40°C. Each batch was discarded after 1 month.

2.3.3. Analysis of colony cell types

Colony type was verified periodically by transferring colonies from the plates into 200μl cooled IMDM with 30% FCS using an automatic pipette. The cells were gently agitated and then transferred onto a glass slide by cytocentrifugation (300g for 3 min). The slides were allowed to air dry, fixed as appropriate, stained with May Grunwald Geimsa or combined esterase (Naphthol AS-D Chloroacetate and α-naphthol acetate) and examined under the light microscope.

For May Grunwald Giemsa staining the slides were fixed in methanol for 10 min, stained in 50% May Grunwald for 15 min, transferred to 10% Giemsa stain for 10 min and finally washed in buffered water before air drying.

For combined esterase staining the slides were fixed in phosphate buffered acetone formaldehyde at 6°C for one

minute, stained with a phosphate buffered solution containing ethylene glycol monomethyl ether (1/25 vol/vol), 20mg α-naphthol acetate and 15mg fast garnet GBC for 30 min at room temperature, washed and transferred to phosphate buffered solution containing the filtered products of N,N dimethylformamide mixed with naphthol AS-D chloroacetate and fast blue BB for a further 30 min before being washed and counterstained with Harris haematoxylin for 5 min and air dried.

2.3.4. Expression of CFU-GM results

CFU-GM were expressed per ml of blood. This value was obtained by comparing the numbers of colonies obtained for a known MNC plating number with the total MNC count/ml obtained from a full blood count sample taken at the same time.

2.3.5. Blood cell counting

Platelets and white cells were counted by a Sysmex NE8000 (LEP Scientific) automated cell counter. Where required visual 100 cell differential white cell counts were performed.

2.3.6. Statistics

Comparisons were made using the student's t-test on square root transformed data.

2.4 Results

2.4.1 Morphology of CFU-GM colonies

The characteristic appearance of a CFU-GM colony is shown in fig 2.1. After removal from the culture well and staining, the granulocytic and monocytic elements can be seen (fig 2.2 and 2.3).

2.4.2 Effect of MNC plating concentration

The effect of plating an increasing concentration of MNC on CFU-GM colony numbers is shown in figure 2.4 and 2.5. A linear increase in CFU-GM was found with MNC from normal individuals and from PBSCH.

2.4.3. CFU-GM in a normal population

CFU-GM assays performed on the peripheral blood of 43 healthy volunteers resulted in a mean value of 166 CFU-GM/ml, SEM 19. The data was normally distributed once square root transformed. A group of 20 values obtained near the beginning of the experimental period was not significantly different from a group obtained towards the end of the period ($p = 0.38$).

2.4.4. CFU-GM in patients with lymphoma

2.4.4.1. NHL

Patients with NHL received the chemotherapy regimes shown in table 2.III.

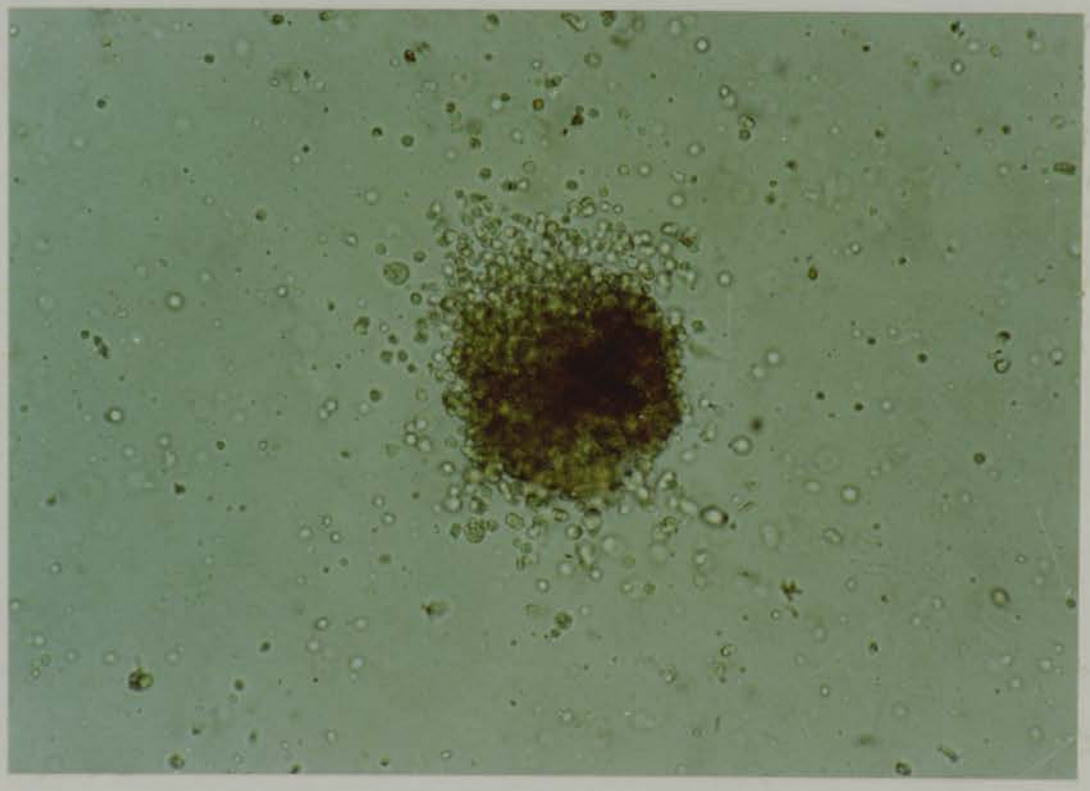

FIGURE 2.1 Photomicrograph of CFU-GM colony in situ in culture well (x40)

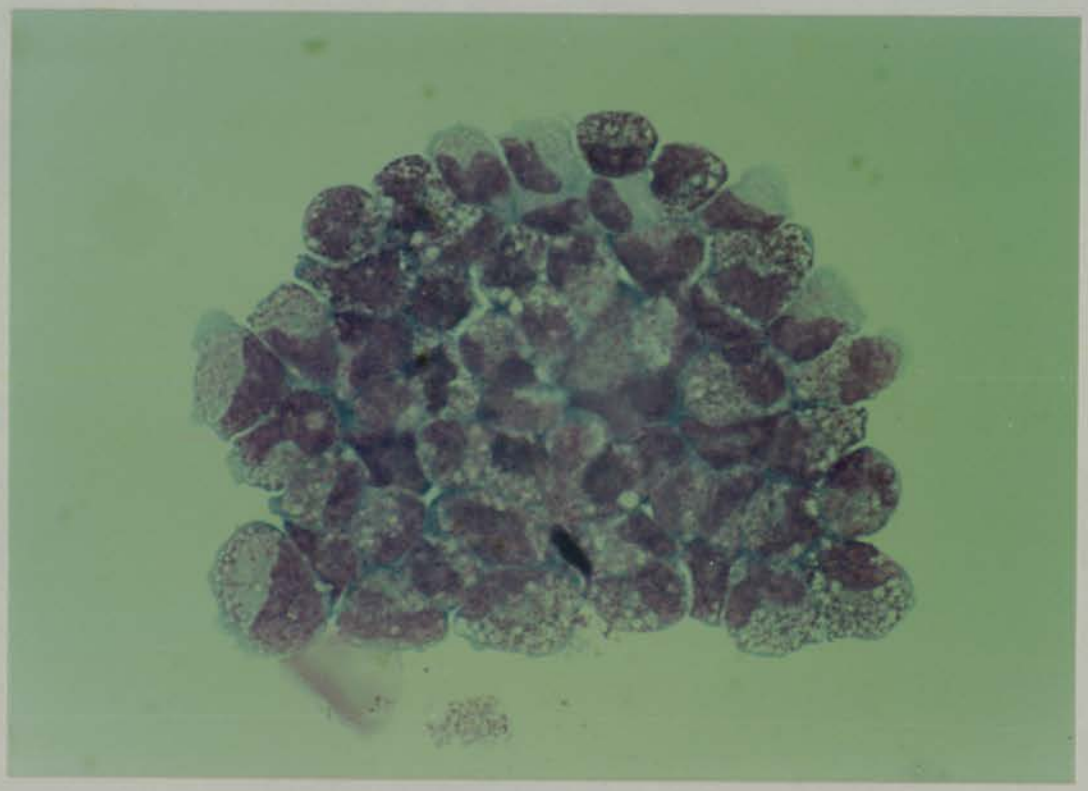

FIGURE 2.2 Photomicrograph of cytospin preparation of cells from a CFU-GM colony stained with May Grunwald Giemsa (x80) showing cells of the granulocyte and monocyte lineages.

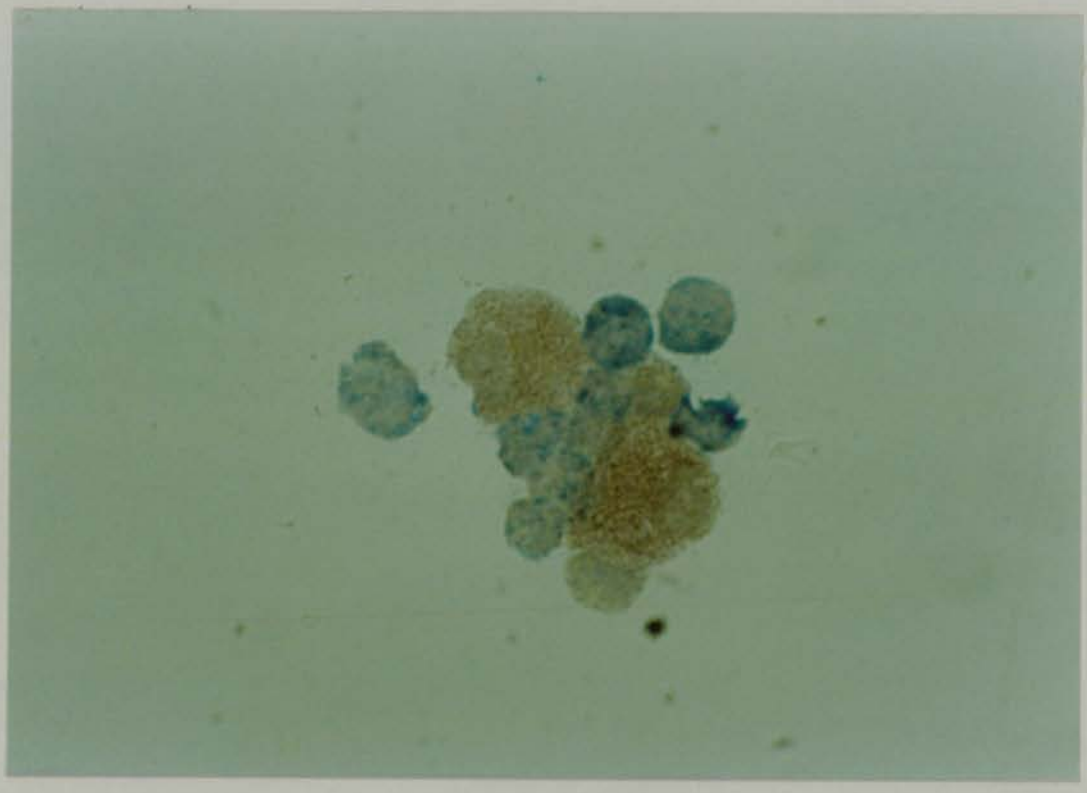

FIGURE 2.3 Photomicrograph of cytospin preparation of cells from a CFU-GM colony stained with combined esterase and demonstrating the presence of both cell types. Granules in the cytoplasm of cells of the monocyte lineage stain brown whilst granules in the cytoplasm of cells of the granulocyte lineage stain blue.

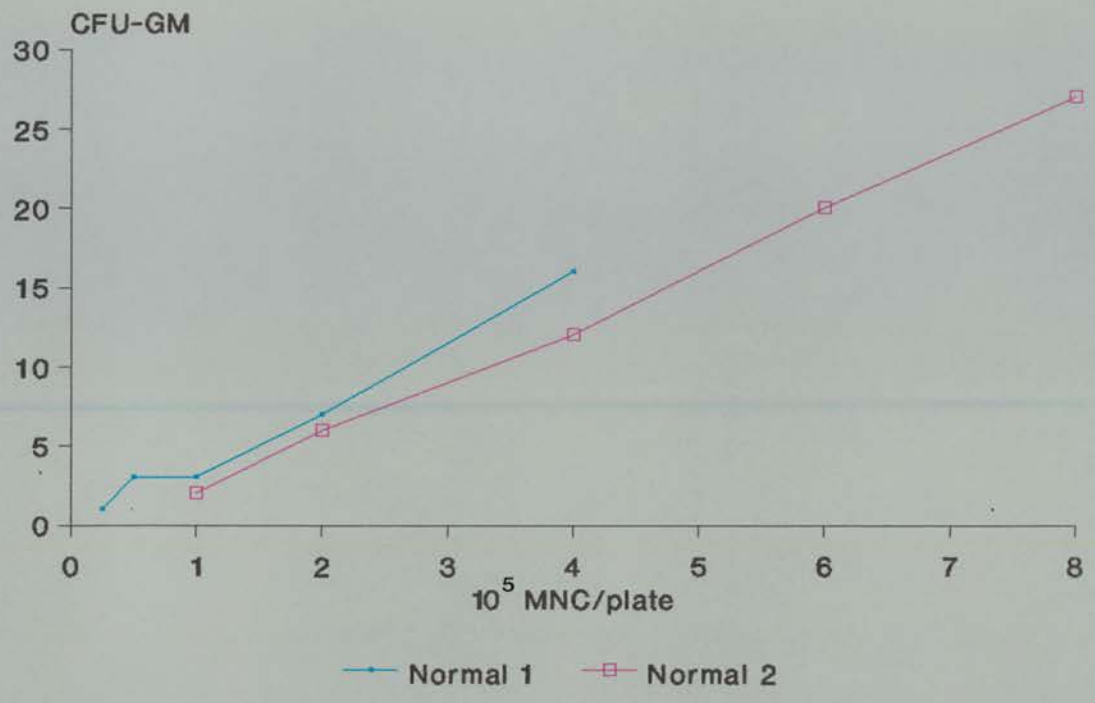

FIGURE 2.4 The effect of increasing MNC plating concentration on the absolute number of CFU-GM colonies in the peripheral blood from 2 normal volunteers.

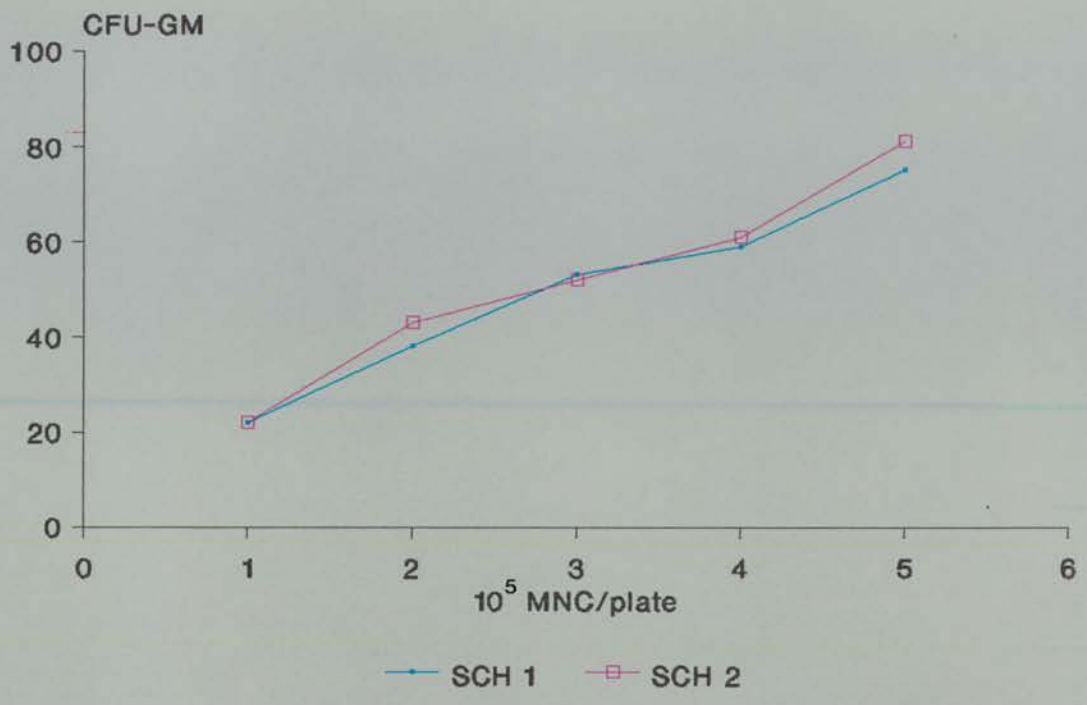

FIGURE 2.5 The effect of increasing MNC plating concentration on the absolute number of CFU-GM colonies from 2 stem cell harvests.

Two patients were treated with BCHOP alternating with PEEC whilst the others received only one regime.

The maximum levels of CFU-GM/ml blood throughout the whole chemotherapy period irrespective of course number, are shown in fig 2.6. CVP and VAD raised CFU-GM by 2.9 and 2.4 fold respectively, whereas PEEC produced wide variations in values in the 2 patients studied. ALL therapy induced a mean 15.6 fold increase in PBSC occurring between days 20 - 22. In the 18 patients who received CHOP based regimes the mean CFU-GM peak was 1026/ml representing a 6.2 fold rise over our normal mean value and occurred on average at day 19 (range 15 - 29) (table 2.V). In one patient, CFU-GM did not rise. An example of the response of CFU-GM to BCHOPM chemotherapy in a typical patient with NHL is shown in figure 2.7.

2.4.4.2. Hodgkin's Disease

Figure 2.8 shows the maximum circulating CFU-GM in 22 patients with HD treated with various chemotherapy regimes. Six patients received alternating regimes (5 MOPP/ABVD, one HOPE/ChlVPP) and one received both first line and second line chemotherapy. No increase was found in 45% of patients including 4 of 10 treated with ChlVPP, 2 of 5 after MOPP and 3 of 9 after ABVD. These regimes produced a 2.8 - 3.5 fold increase in CFU-GM over normal values. HOPE induced a mean 14.5 fold rise in all 4 patients receiving this regime. (table 2.VI).

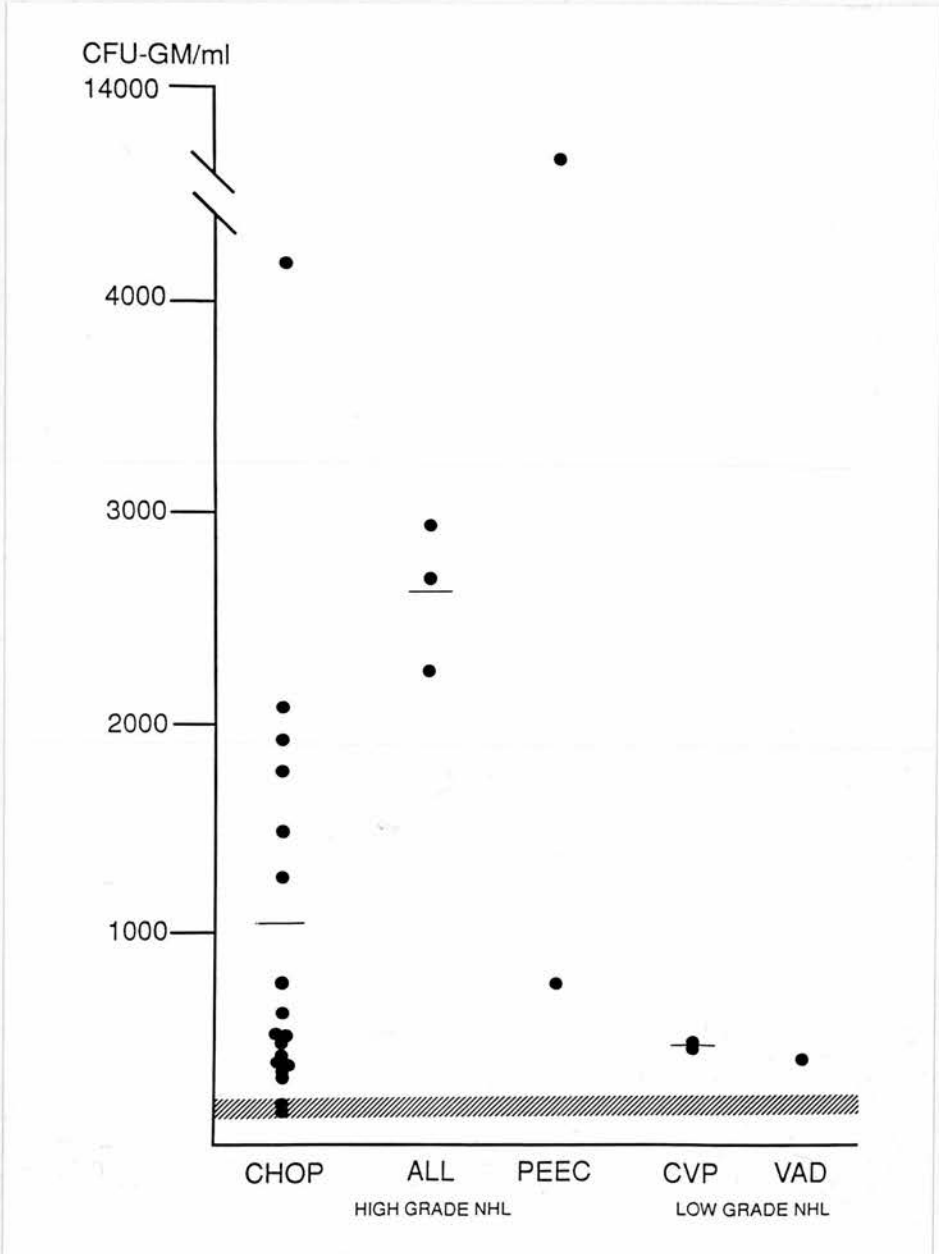

FIGURE 2.6 Peak levels of CFU-GM/ml in 24 patients with NHL treated with various chemotherapy regimes. The mean values obtained after each chemotherapy regime is shown as a dotted line and the mean CFU-GM ± one SEM found in our normal population represented in the hatched area.

Chemotherapy Regime		n	Mean Peak CFU-GM/ml (range)	Mean day of peak (range)
NHL	**CHOP**	18	1026 160-4540	19 15-29
	ALL	3	2595 2227-2906	21.3 20-22
	PEEC	2	7181 768-13595	18.5 15-22
	CVP	2	475 461-490	11 9-13
	VAD	1	391	22

TABLE 2.V Response of CFU-GM to chemotherapy in patients with NHL.

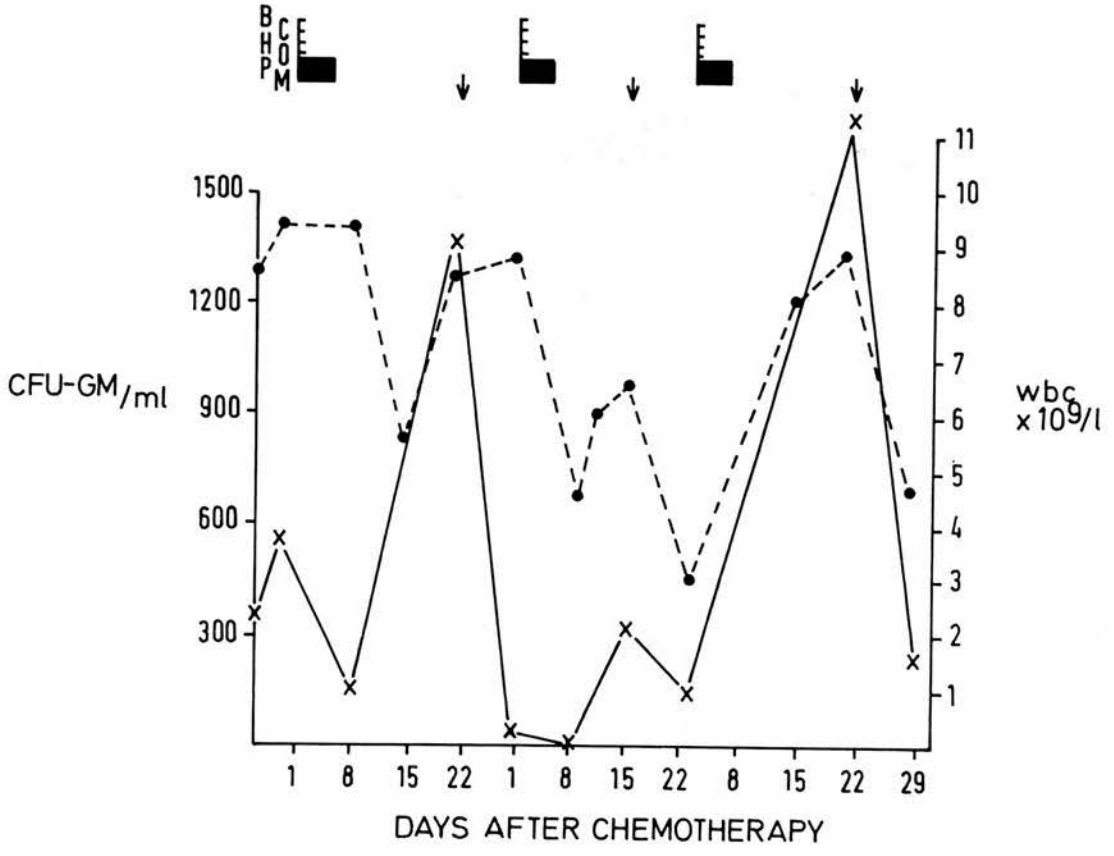

FIGURE 2.7 Response of CFU-GM (solid line) and white blood count (wbc, dotted line) after 3 courses of BCHOPM chemotherapy in a patient wiht high grade NHL.

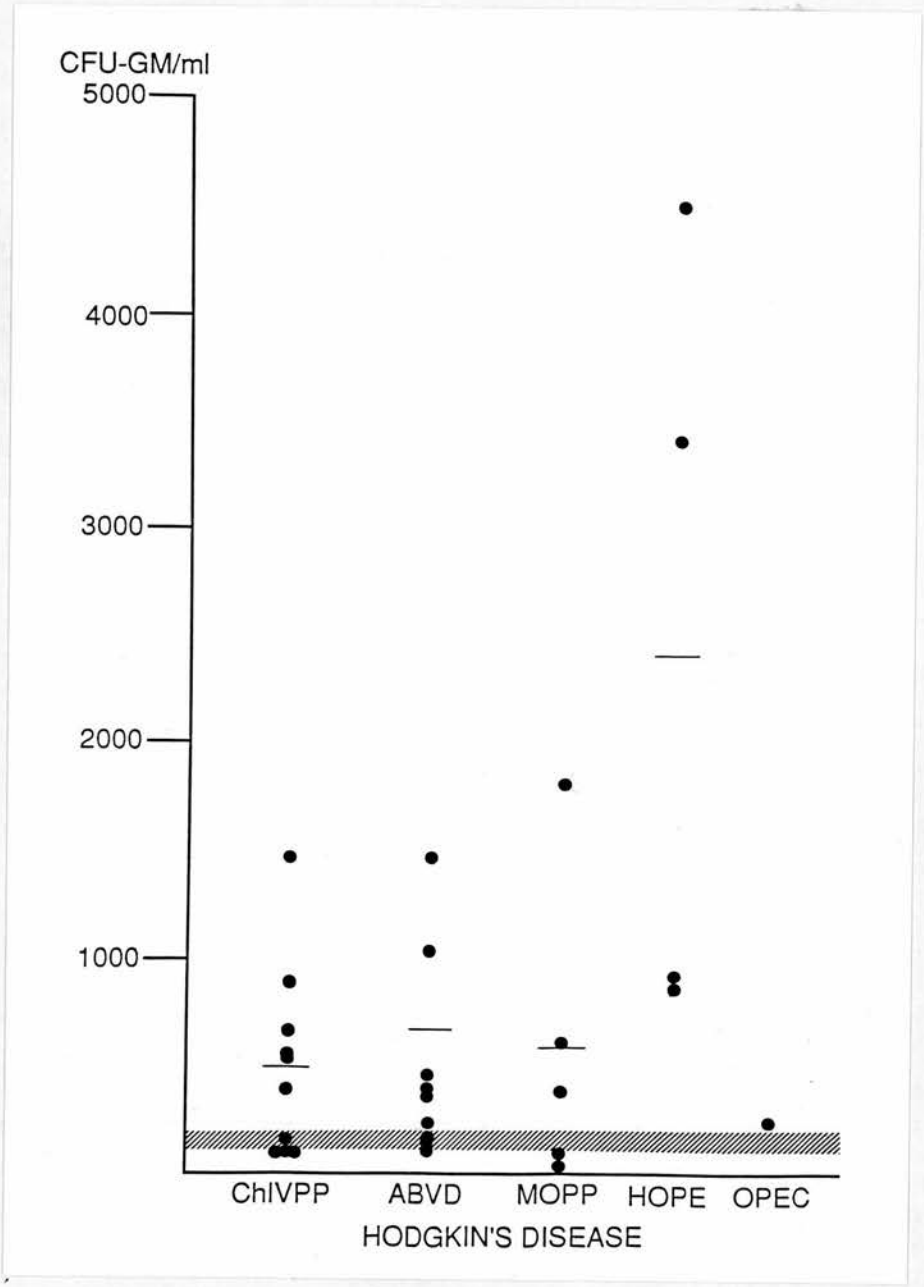

FIGURE 2.8 Peak levels of circulating CFU-GM/ml in 22 patients with HD treated with various chemotherapy regimes. The mean values obtained for each chemotherapy regime is shown as a dotted line and the mean CFU-GM ± one SEM found in our normal population represented by the hatched area.

	Chemotherapy Regime	n	Mean Peak CFU-GM/ml (range)	Mean day of peak (range)
HD	ChlVPP	10	484 78-1461	21.6 15-29
	ABVD	9	670 75-1459	17.4 8-29
	MOPP	5	577 20-1805	30.4 22-50
	HOPE	4	2410 867-4479	25 22-34
	OPEC	1	238	30

Table 2.VI Response of CFU-GM to chemotherapy in patients with HD

2.4.4.3. Effect of marrow involvement

Nine patients (3 HD, 6 NHL) had bone marrow involvement by disease detected by morphology and/or immunophenotyping. CFU-GM increases (469/ml ± 76) were significantly less ($p = 0.017$) in these patients when compared to those with no evidence of marrow infiltration (1319/ml ± 339) (fig 2.9). Of the 18 patients with NHL treated with CHOP based regimes, 3 had bone marrow involvement with lymphoma. These 3 patients had significantly lower increases in CFU-GM (451/ml ± 21) compared to those without marrow involvement (1141/ml ± 292; $p = 0.024$).

2.4.4.4. Effect of second line chemotherapy

Eight patients (7 HD, 1 NHL) had relapsed disease and were receiving second or subsequent line chemotherapy. A significantly lower CFU-GM peak (295/ml ± 48 cf 1328/ml, $p = 0.0001$) was found in those treated with second or subsequent therapies (fig 2.10).

2.4.4.5. Effect of course number

In those patients with NHL receiving CHOP based regimes on whom there was adequate data during chemotherapy, no significant difference was found in the mean CFU-GM peak after successive treatment courses. (Table 2.VII)

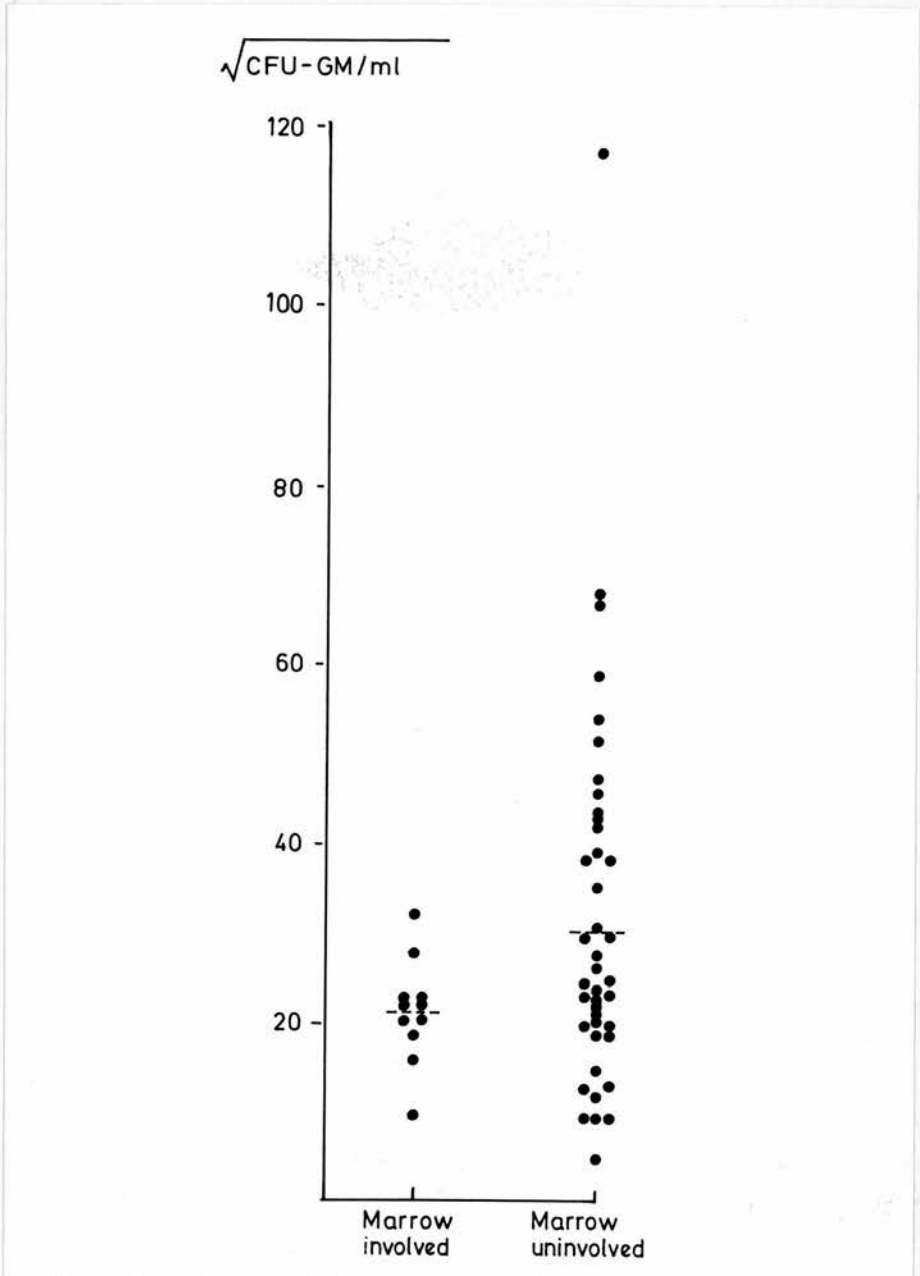

FIGURE 2.9 Peak levels of CFU-GM after chemotherapy in patients with lymphoma with bone marrow involvement compared to those without bone marrow involvement by disease. Significantly lower peak CFU-GM/ml were found in those patients with marrow involvement (Student's t-test on square root transformed data, $p = 0.017$).

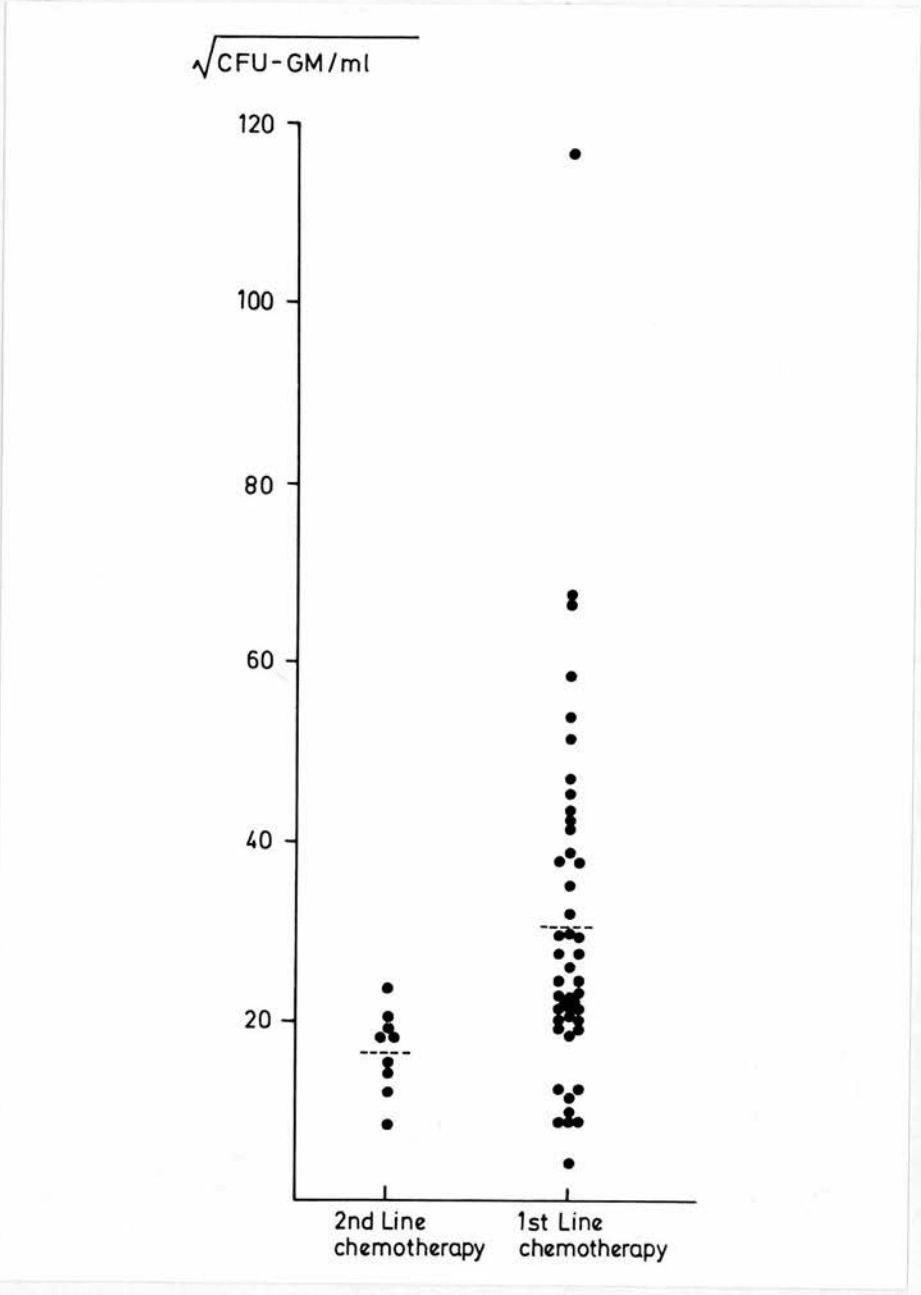

FIGURE 2.10 Peak levels of CFU-GM/ml in patients with lymphoma in those receiving first line chemotherapy compared with those receiving second or subsequent line chemotherapy. Significantly lower levels of CFU-GM/ml were found in those patients receiving second line or subsequent line chemotherapy (Student's t-test on square root transformed data, p = 0.0001).

Course Number	n	Mean Peak CFU-GM/ml	Mean Day of Peak	Comparison to 1st course
1	13	923	20.5	
2	15	553	19.4	p = 0.34
3	7	204	21.6	p = 0.064
4	6	440	20.5	p = 0.26

TABLE 2.VII Effects of successive courses of CHOP chemotherapy on CFU-GM peak.

2.4.5. CFU-GM in acute leukaemia

2.4.5.1 AML

Figure 2.11. shows the peak circulating CFU-GM in 9 patients with AML treated with either DAT (5 patients) or ADE (4 patients) chemotherapy. Three patients went on to receive a second course of DAT and 2 a second course of ADE. The mean CFU-GM peak in those treated with DAT was 6336/ml, a 38 fold rise over normals occurring on average at day 28.6 (range 20 - 36). Those receiving ADE demonstrated a 12.5 fold increase to 2083 CFU-GM/ml on day 26 (range 23 -28) (Table 2.VIII). The response of CFU-GM to ADE chemotherapy in a typical patient is shown in fig 2.12. One patient in each treatment group did not have a rise in PBSC. Neither demonstrated a platelet count above 60, nor had they entered remission after this course of treatment. There was no significant difference in the levels of CFU-GM attained after the first or second course of treatment in the small group of 5 patients as a whole, however, when considered individually variations in CFU-GM response were found Fig 2.13.

2.4.5.2. ALL

Four patients with ALL received treatment according to the UKALL X protocol (table 2.IV) and the peak CFU-GM/ml obtained shown in fig 2.11. After induction therapy a 5.3 fold rise in CFU-GM occurred on average on day 24 (range 21 - 29). Intensification, however produced considerably greater increases in PBSC to 5882/ml (a 35 fold rise).

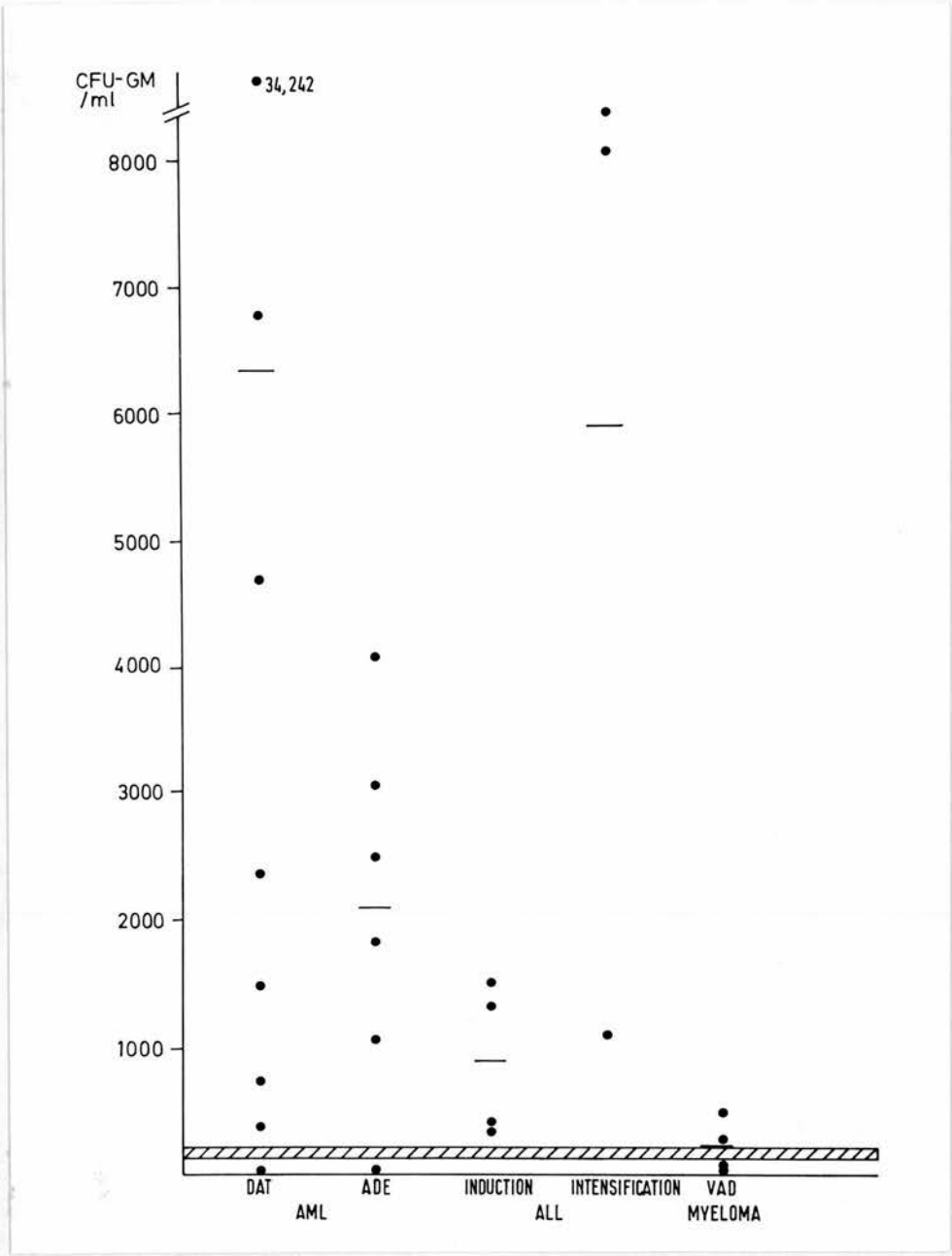

FIGURE 2.11 Peak levels of CFU-GM/ml in patients with acute leukaemia and myeloma treated with various chemotherapy regimes. The mean values obtained after each chemotherapy regime is shown as a dotted line and the mean ± one SEM found in our normal population is represented by the hatched area.

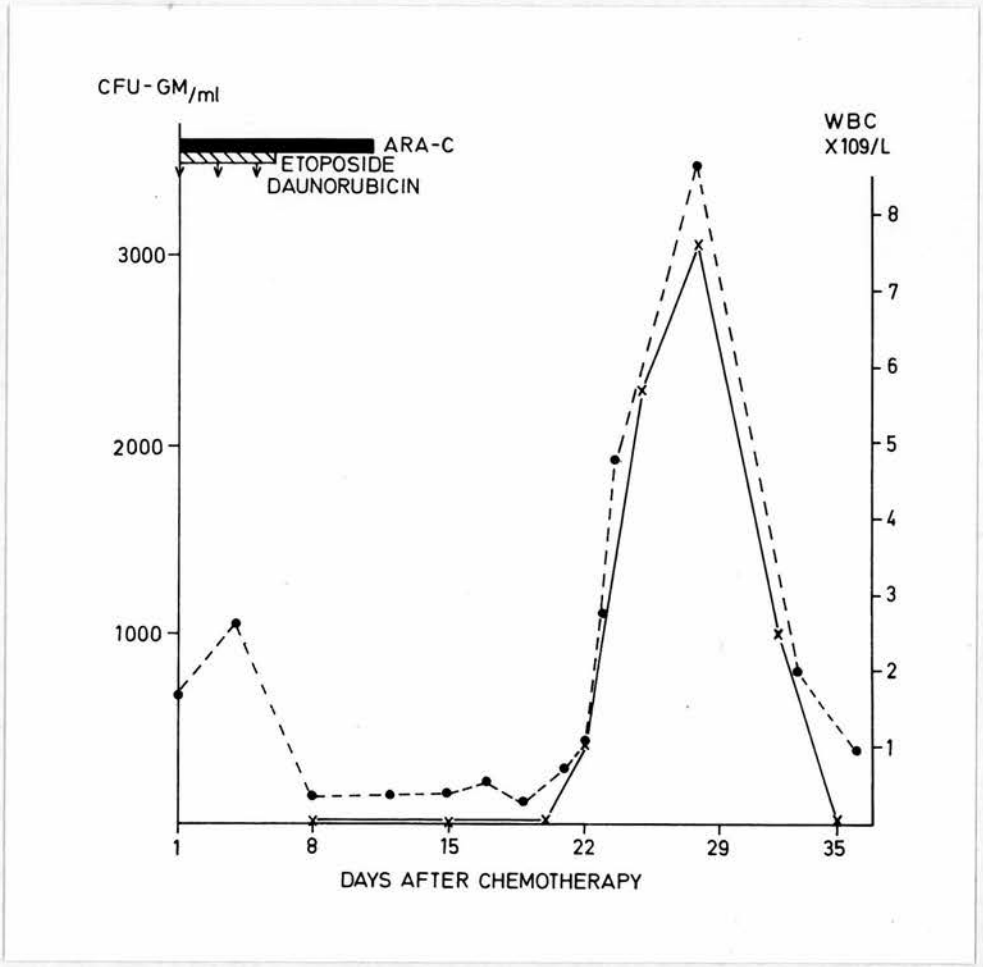

FIGURE 2.12 The reponse of CFU-GM (solid line) and white blood count (WBC, dotted line) to ADE chemotherapy in a typical patient with AML

Chemotherapy Regime	n	Mean Peak CFU-GM/ml (range)	Mean day of peak (range)
DAT	5	6336 0 - 34 243	28.6 20 - 36
ADE	4	2083 0 - 4054	26 23 - 28
ALL Induction	4	886 383 - 1493	24 21 - 29
ALL Intensification	3	5882 1088 - 8013	26 17 - 39
MACHO (Ara-C)	1	2784	19

Table 2.VIII The response of CFU-GM to chemotherapy for acute leukaemia

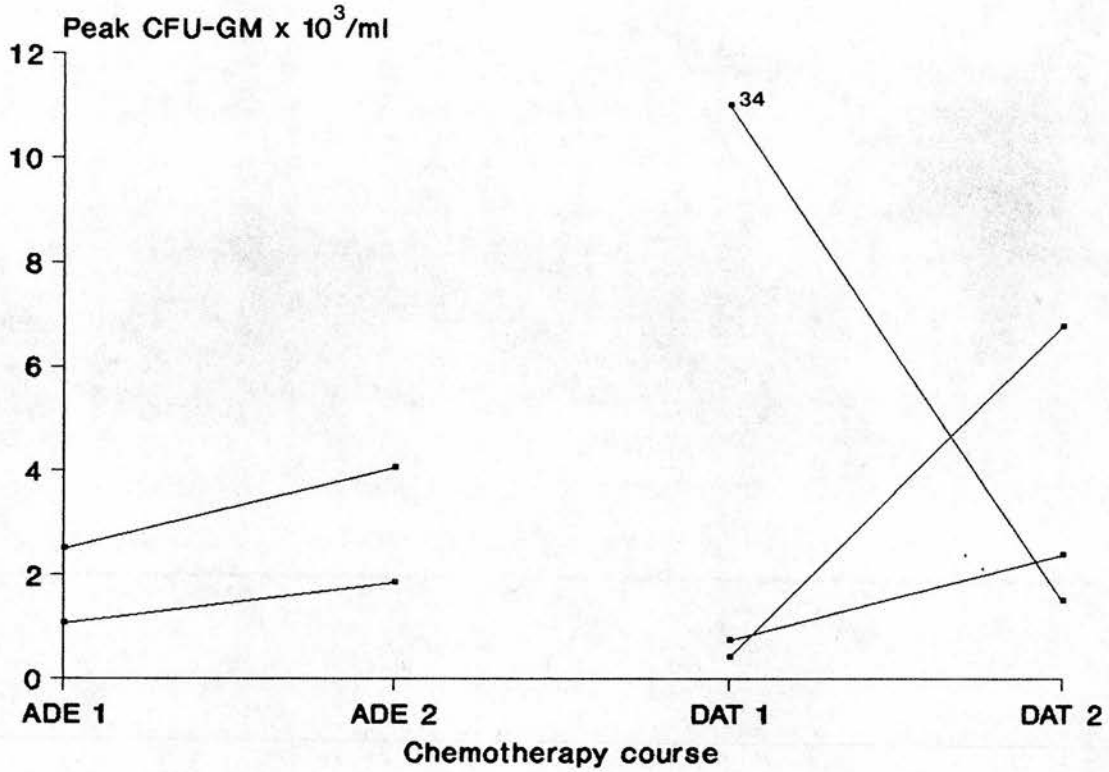

FIGURE 2.13 The effect on peak CFU-GM/ml of succesive courses of ADE in 2 patients and of successive courses of DAT in 3 patients with AML.

The one patient treated with MACHO increased CFU-GM to 16.8 fold 19 days after receiving high dose Ara-C.

2.4.5.3. Predictors of CFU-GM peaks

In the leukaemic group as a whole, the CFU-GM elevation started when the mean wcc was 1.5 x 10^9/l and the peak occurred when the mean wcc was 5.9 x 10^9/l. The mean percentage of monocytes at the time of peak was elevated at 10.2% (range 1 -29%) (table 2.IX). Although there was no statistically significant correlation between the peak CFU-GM and the peak platelet count, it was noteable that the 2 patients who failed to increase PBSC did not reach a platelet count above 60 x 10^9/l. The rate of recovery of the wcc did compare with the level of CFU-GM obtained: those patients who took longer than 5 days to increase the wcc from 1 to 3 x 10^9/l had a significantly lower CFU-GM peak than those who took 5 days or less ($p = 0.037$, fig 2.14).

2.4.6. Myeloma

Four patients with myeloma received VAD chemotherapy for their disease. Three patients had previously received other chemotherapy regimes. The mean CFU-GM peak was only 190/ml and only one patient increased PBSC more than twice normal.

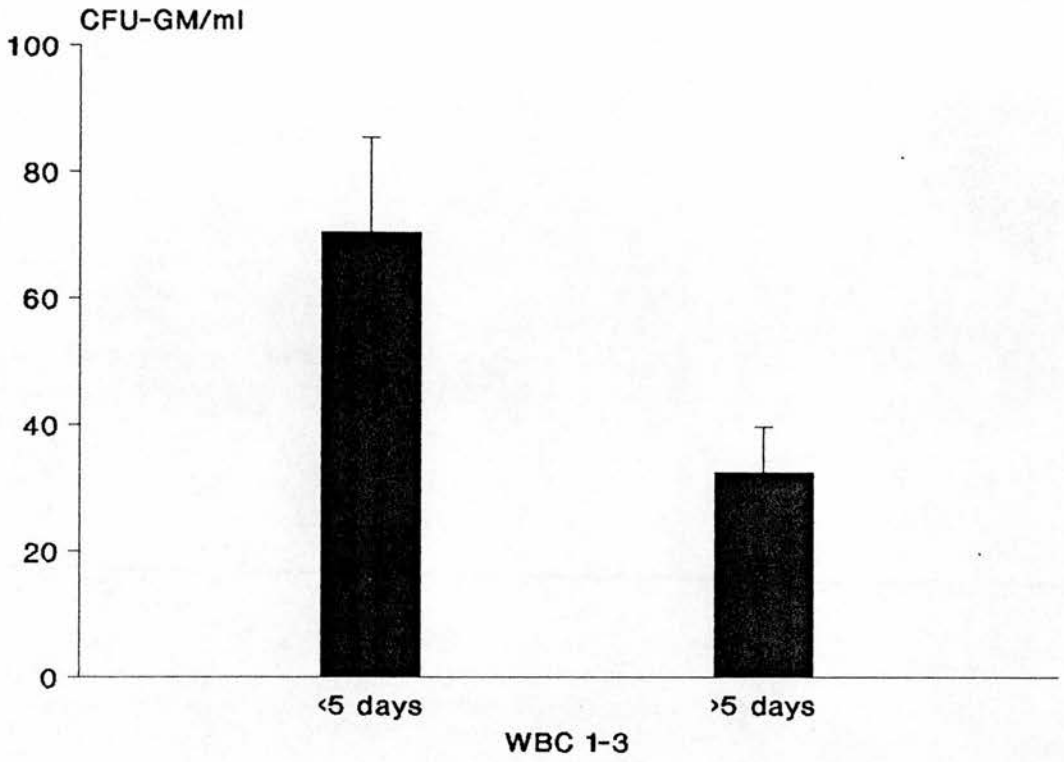

FIGURE 2.14 The peak value of CFU-GM is related to the rate of recovery of the white cell count. The peak values of CFU-GM/ml reached in those patients with acute leukaemia whose white blood count rose from 1-3 x 10^9/l in 5 days or less (wbc 1-3 < 5 days) were significantly greater than the peaks attained by those whose WBC took more than 5 days to recover from 1 - 3 x 10^9/l (WBC > 5 days), (Student's t-test on square root transformed data, p = 0.037). The data shown represent the mean peak CFU-GM/ml ± one SEM.

	mean	range
WBC at start $\times 10^9/l$	1.5	0.2 - 3.0
WBC at peak $\times 10^9/l$	5.9	1.4 - 25.6
Platelets at peak $\times 10^9/l$	212	27 - 670
Monocytes at peak %	10.2	1 - 29

TABLE 2.IX Blood parameters during CFU-GM elevations

2.5. Conclusion

Standard chemotherapy can induce a rise in PBSC in 85% of patients with lymphoma and 85% of patients with acute leukaemia at predictable times. Significantly lower peak values were found in those lymphoma patients who had received previous chemotherapy or who had marrow involvement.

CHAPTER 3

PERIPHERAL BLOOD STEM CELL HARVESTING

Having determined the pattern of stem cell response to standard chemotherapy regimes, the feasibility of harvesting these progenitors was assessed.

3.1. Materials

ACD-A (Scottish National Blood Transfusion Service, Edinburgh)
DMSO (Sigma, Poole, Dorset)
DNAse (Sigma)
Ficoll-metrizoate (Nycomed, Oslo, Norway)

3.2. Patients

After obtaining informed consent and medical assessment, 74 PBSC harvests were obtained from 25 patients with various haematological malignancies (Table 3.I) at a time when counts were rising or when previous studies had indicated PBSC would be raised.
AML was classified according to the FAB criteria (Bennett et al, 1985), ALL according to MIC criteria (First MIC Cooperative Study Group, 1986), and NHL according to the Keil classification (Stansfield et al, 1988). Patients with lymphoma were clinically staged according to the Ann Arbor system (Carbone et al, 1971).
Of the patients with NHL, 1 was stage I and 7 stage IV.

The majority of those with HD also had advanced disease, 5 were stage IV, 2 stage III and one stage I. Patients with AML consisted of 2 M_1, one M_2, one M_3 and one M_5. Two patients had common ALL and one B cell ALL.

DISEASE	TARGET MINIMUM CFU-GM x 10^4/kg	START	TIMING AND NUMBER OF PROCEDURES
Acute Leukaemia	30	wcc 1.5x10^9/l	after induction and intensification. Harvest daily or every other day. Aim for 5 harvests each time.
Lymphoma	5	during week of haematopoietic recovery	2-3 harvests after each course of chemotherapy. If no increase in CFU-GM after 2 courses, stop.

TABLE 3.A CRITERIA FOR PBSCH

	n	M/F	age(yr)	range	n PBSCH
NHL	8	4M/4F	43.5	24-56	21
HD	8	7M/1F	32.6	14-57	33
AML	5	3M/2F	31.6	17-46	10
ALL	3	3M	23	17-30	9
Myeloma	1	1M	61		1

TABLE 3.1 Characteristics of patients undergoing PBSCH

3.3. Methods

3.3.1. Peripheral Blood Stem Cell Harvests

Harvests were performed using the Fenwall CS3000 blood cell separator set to standard procedure 3 (lymphocyte collecting procedure) with the interface detector set at 20, centrifuge speed 1400 rev/min and blood flow rate 50ml/min . ACD-A at a ratio of 1:13 with whole blood was used as anticoagulant. In flow and out flow venous access lines were established using 14 F gauge cannulae in peripheral veins or long term indwelling central venous catheters. A target of 9 litres of blood was processed. Throughout the procedure the patients were closely monitored by trained nursing staff. CFU-GM and blood counts were performed as previously described (2.3.1. and 2.3.5.) before and after each harvest and also on the product obtained. Collecting efficiencies were evaluated by calculating the number of circulating cells/ml x volume blood processed divided by the number of cells obtained in the harvest x 100.

3.3.2. Secondary Processing and Cryopreservation

In order to reduce red cell contamination and volume of the stem cell harvest prior to cryopreservation, secondary processing was performed using an IBM 2991 cell processor. The method was based on that of Gilmore et al (1982) for processing bone marrow. The PBSCH was layered under pressure onto 150ml ficoll-metrizoate and centri-

fuged at a relative centrifugal force between 905 and 1006 for 25 min. The MNC layer was then collected and washed twice in 0.9% saline with 20% autologous plasma to remove the ficoll-metrizoate before being diluted 1:1 with 20% DMSO in autologous plasma, transferred to 2 Gambro bags, frozen using a controlled rate freezer (Planer PTC 200) and stored at -196°C in the vapour phase of liquid nitrogen. Five hundred microlitre aliquots of the final mixture were withdrawn as pellets and frozen and stored in a similar manner.

3.3.3. Assessment of Pellet Viability

Cell pellets were thawed rapidly at 37°C (2-3 min) and immediately mixed with FCS containing 400u/ml DNAse, 100u/ml preservative free heparin and 0.015M $MgSO_4$ and left at room temperature for 10 min. The cells were washed initially in IMDM, then in IMDM + 5 % FCS and finally resuspended in IMDM + 2% FCS before being cultured in the CFU-GM assays as previously described (2.3.1.). CFU-GM recoveries were assessed at 1, 3, 6, 9, 12 and 18 months after collection.

3.4 Results

3.4.1. PBSCH Product

Seventy four PBSCH were obtained from 25 patients with haematological malignancies. A mean of 7.72 litres of blood were processed (range 2.3 - 91) taking between 65 to 200 min (mean 162 min). The contents of the 200ml product is shown in table 3.II.

The median neutrophil contamination in the PBSCH was 1%, with 11 harvests containing greater than 5% neutrophils. The collecting efficiency for CFU-GM was 65.7% (range 0 - 293) and for MNC 55.6% (range 7 - 110). There was no correlation between the numbers of CFU-GM and MNC collected (r = 0.13).

3.4.2. Effects of PBSCH on patients

The harvest procedure was tolerated well by the majority of patients and was performed as an out-patient in 51% of cases. Poor venous flow was a problem in approximately 15% but only 3 collections were prematurely stopped. The use of a central venous line for either the venous outlet or return improved patient tolerance. Two patients had a vasovagal episode, one confessed to omitting breakfast after a late-night! Both were able to continue with the harvest on recovery.

Peripheral blood platelets were reduced by a mean of 41% (range 4-73%) and CFU-GM by 29%, although in 9 patients CFU-GM were increased by the harvest. MNC were reduced on average by 19%

	mean	range
haematocrit (%)	16.9	2.1-25
MNC ($x10^9$)	5.6	1 -21.5
CFU-GM ($x10^6$)	3.0	0-127
Platelets ($x\ 10^{11}$)	2.46	0.1 - 5.61

Table 3.II PBSCH product

3.4.3. Effects of secondary processing.

After the PBSCH was processed on the IBM 2991, the volume was reduced to 75ml and the haematocrit to 0.9%. However, the mean recovery of CFU-GM was 72% (range 0 - 246).

3.4.4. PBSCH in patients with lymphoma

The details of the harvests from patients with lymphoma are shown in table 3.III. Stem cell harvests yielded a mean of 0.99 x 10^9MNC/kg containing 2.99 x 10^4 CFU-GM/kg per procedure. No correlation was found between the number of MNC and CFU-GM harvested. In patients who were harvested after second or subsequent line chemotherapy significantly lower numbers of CFU-GM were obtained than from those receiving first line treatment ($p = 0.012$, Student's t-test on square root transformed data)(Fig 3.1).

Patient	Chemotherapy		no. of	MNC x 10^8/kg		CFU-GM x 10^4/kg	
	Regime	Line	PBSCH	Total	Mean/procedure	Total	Mean/procedure
NHL							
MA	ALL	1st	2	1.18	0.59	10.2	5.1
MAP	ALL	1st	5	2.89	0.58	29.3	5.86
RB	CHOP	1st	3	3.84	1.28	8.46	2.82
TH	CHOP	1st	4	5.81	1.45	12.3	3.11
LW	CHOP	2nd	2	0.8	0.4	4.77	2.38
AM	CHOP	2nd	3	3.09	1.03	6.67	2.22
SP	CHOP	1st	1	0.91		1.34	
SM	CHOP	1st	1	1.48		1.18	
HD							
BP	MOPP	1st	3	3.82	1.27	49.3	16.4
GM	MOPP	2nd	2	0.83	0.41	0.35	0.17
DB	ClVPP	2nd	2	2.55	1.27	2.13	1.1
DO	OPEC	3rd	4	4.4	1.1	0.92	0.23
EN	ABVD	2nd	1	0.36		0.18	
KF	ABVD	2nd	7	9.2	1.31	1.6	0.23
GL	ABVD	1st	2	3.84	1.92	7.64	3.82
AS	ABVD	1st	12	6.69	0.67	9.08	0.91

TABLE 3.III Stem cell harvests from patients with lymphoma.

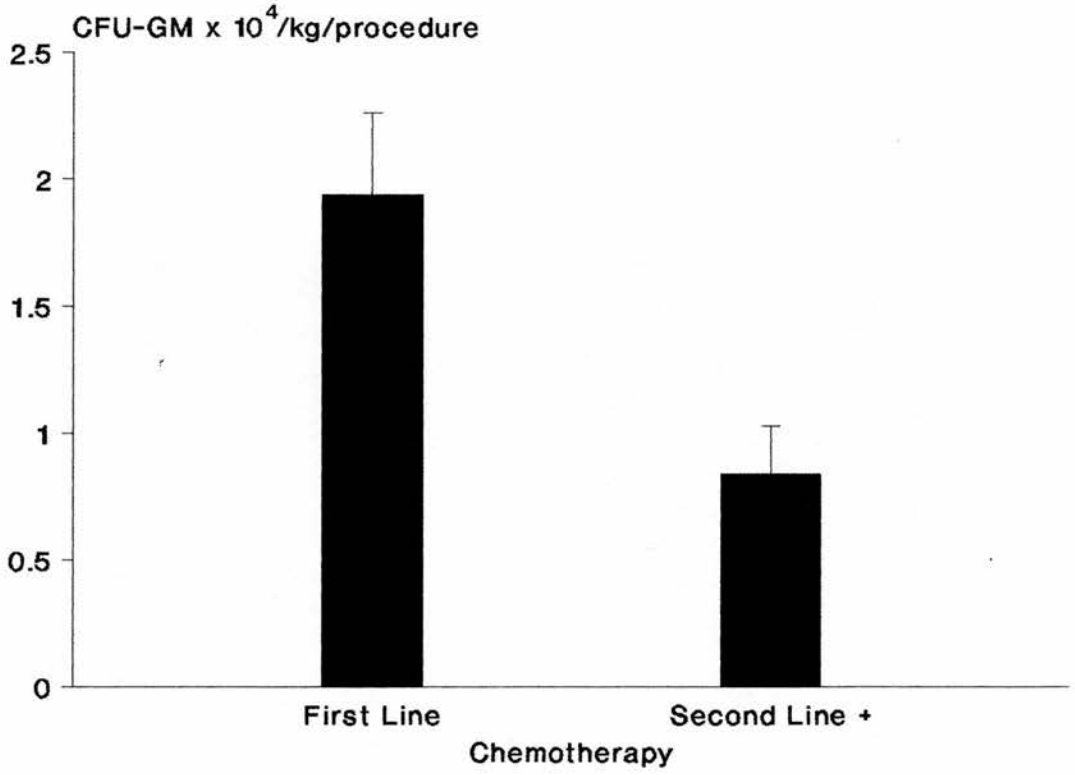

FIGURE 3.1 CFU-GM harvested from patients after first line or after second or subsequent line chemotherapy. Significantly lower numbers of CFU-GM were obtained in those receiving second line or greater therapy than in those patients harvested during first line treatment ($p = 0.012$, Student's t-test on square root transformed data).

3.4.5. PBSCH in patients with acute leukaemia and myeloma

Table 3.IV shows the details of stem cell harvests obtained from patients with acute leukaemia and one patient with myeloma. A mean of 1.2×10^8 MNC/kg with 8.6×10^4 CFU-GM/kg per procedure were harvested from those with acute leukaemia.

Patient	Chemotherapy		no. of	MNC x 10^8/kg		CFU-GM x 10^4/kg	
	Regime	Line	PBSCH	Total	Mean/procedure	Total	Mean/procedure
AML							
KD	DAT	2nd	2	0.43	0.21	1.11	0.55
FC	MACE	1st	3	4.0	1.33	4.68	1.56
SW	MidAc	1st	2	3.64	1.82	6.14	3.07
DC	ADE	1st	2	2.88	1.44	56.4	28.2
JH	MACE	1st	1	2.43		7.2	
ALL							
GG	MACHO	1st	1	0.6		0.7	
DP	UKALL	1st	5	2.68	0.54	74.6	14.9
WL	UKALL	1st	3	3.71	1.24	38.8	12.9
MYELOMA							
JB	VAD	2nd	1	1.57		0.71	

TABLE 3.IV Stem cell harvests from patients with acute leukaemia and myeloma.

3.4.6. Pellet Viabilities

CFU-GM recoveries were assessed on 33 PBSCH pellets after 1 month of storage. The mean recovery was 157% of the CFU-GM in the pellets before freezing. After 3 months 40 pellets showed a recovery of 200%, after 6 months 38 pellets showed 138% recovery, after 9 months 25 pellets showed 102% recovery, after 1 year 23 pellets showed 70% recovery and after 18 months 8 pellets showed 50% recovery (Fig 3.2). There was no significant difference in the % recovery at one month compared with that at 3, 6 and 9 months but the recoveries at one year and 18 months were significantly lower ($p = 0.018$ and $p = 0.0039$ respectively).

Figure 3.3 illustrates the comparison of growth in the pellet to that found in the PBSCH Gambro bag at time of PBSC transplant and shows that in all but patient 7, the CFU-GM recoveries from the pellets were less than those found in the PBSCH. The lowest CFU-GM recovery in the PBSCH at transplant was 85% (patient 3).

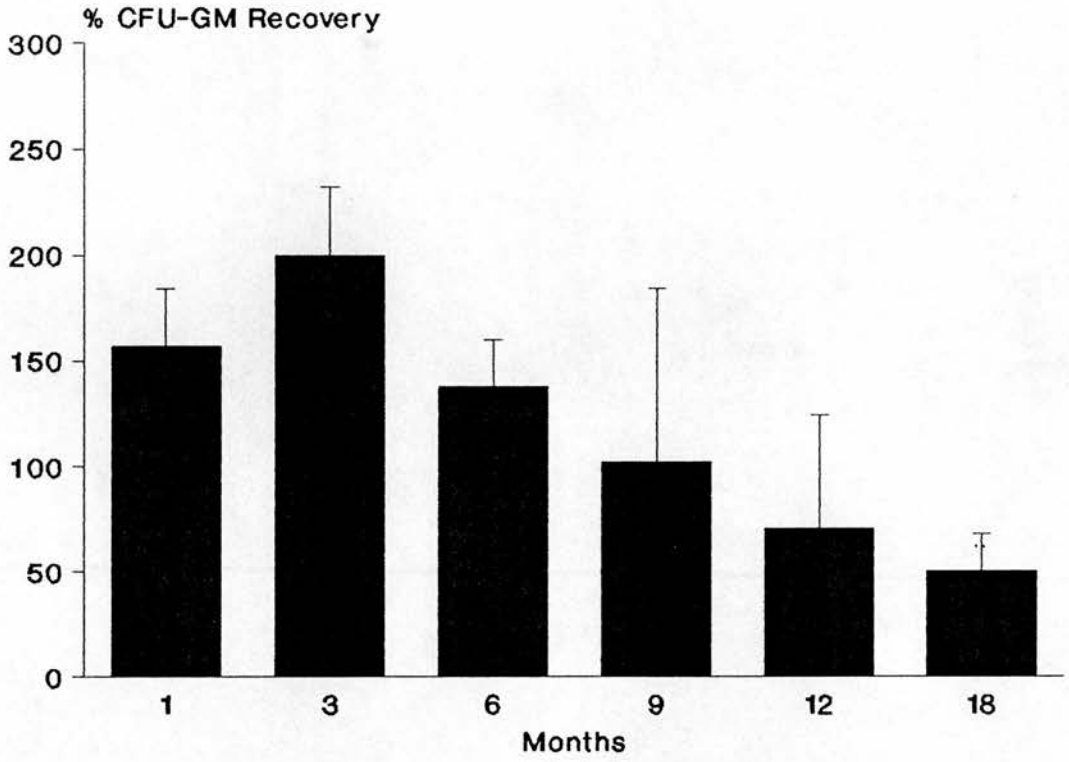

FIGURE 3.2. CFU-GM recoveries from pellets of different PBSCHs after one to 18 months storage. Results are expressed as a percentage of the initial harvest growth. Assessments were performed on the following number of pellets: one month - 33 pellets, 3 months - 40 pellets, 6 months - 40 pellets, 9 months - 25 pellets, one year - 23 pellets, 18 months - 8 pellets.

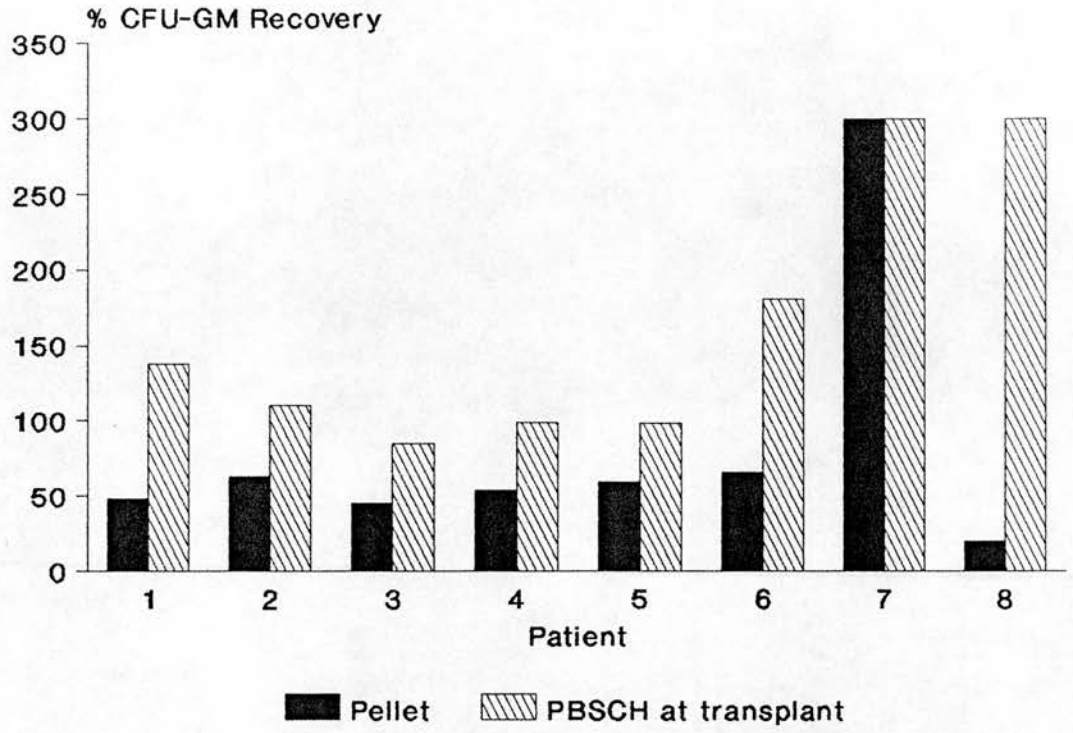

FIGURE 3.3 CFU-GM growth in pellets and the harvest storage bag from 8 patients at the time of PBSC reinfusion. The results are expressed as a percentage of growth obtained at the time of PBSCH and show discrepancies between pellet CFU-GM and growth of PBSCH at time of reinfusion.

3.5. Conclusion

Stem cell harvesting using the Fenwall CS3000 can be efficiently and safely carried out and collect adequate numbers of PBSC if harvests are well-timed. Secondary processing reduced volume and red cell contamination but resulted in a 28% loss of CFU-GM. Monitoring of cryopreserved PBSCH by assessment of pellet growth was unreliable.

CHAPTER 4

GENE REARRANGEMENT STUDIES

Using gene rearrangement studies, the incidence of tumour markers in haematological malignancies and in peripheral blood in patients with NHL at presentation was established. By this method tumour contamination in PBSCH was assessed.

4.1. Materials

DNA Extraction

Red cell lysis solution

0.14M NH_4Cl

0.017M Tris pH 7.65

High TE

0.1M Tris pH 7.65

0.04M EDTA

Lysis solution

0.1M Tris pH 7.65

0.04M EDTA

0.2% SDS

1M NaCl

TE pH 7.6

0.01M Tris pH 7.6

0.001M EDTA pH 8.0

Tris buffered phenol

0.1% 8-hydroxyquinoline (Sigma, Poole, Dorset) in phenol equilibrated with 1M Tris until the aqueous phase was > pH 7.6

RNAse (Sigma, Poole, Dorset, UK) 5mg/ml Boiled for 10 minutes to denature DNase activity

Proteinase K (Sigma) 10mg/ml

Gel electrophoresis

Tris-acetate (TAE)

0.04M Tris-acetate

0.001M EDTA

Stock solution 20x concentrated

Gel Loading Buffer

0.025% bromophenol blue (Sigma)

0.025% xylene cyanol (Sigma)

2.5% Ficoll 400 (Pharmacia, Uppsala)

Stock solution 6x concentrated

Agarose (NA) electrophoresis grade (Pharmacia, Uppsala, Sweden)

Southern blotting

Acid Hydrolysis

0.25M HCl

Denaturing Solution

1.5M NaCL

0.5M NaOH

Neutralising Solution

2M NaCl

1M Tris pH 5.5

Standard Sodium Citrate (SSC)

1.15M NaCl

0.015M tri-sodium citrate

Stock solution 20x concentrated

Ethidium bromide 10mg/ml (Sigma)

Hybridisation

Denhardt's Solution

0.05% Ficoll 400 (Pharmacia)

0.05% polyvinylpyrrolidone

0.05% Bovine serum albumin (BSA) Fraction V (Sigma)

Stock solution 50x concentrated

Prehybridisation fluid

10% dextran sulphate (Pharmacia)

0.1% SDS

0.1% NaPPi

5x Denhardt's solution

5x SSC

Random primed DNA labelling kit (Boehringer Mannheim, Germany)

Nick Column (Pharmacia)

Sheared salmon sperm DNA 20mg/ml (Sigma)

CTP α^{32}P specific activity 3000Ci/mmol (Du Pont de Nemours, Dreieich, W Germany)

Wash solution 1

2x SSC

0.1% SDS

0.1% NaPPi

Wash Solution 2

0.2x SSC

0.1% SDS

0.1% NaPPi

Wash Solution 3

0.1x SSC

0.1% SDS

0.1% NaPPi

Developer solution - GBX developer 20% solution (Sigma)

Fix Solution - GBX Fixer and Replenisher 20% solution (Sigma)

Stop solution - Indicator Stop Bath 4% solution (Sigma)

Nitrocellulose membrane filters (Schleicher and Schuell, Dassel, W. Germany)

Probe stripping solution

0.001M NaOH

0.001M EDTA

All chemicals were analar grade and obtained from BDH (Poole, Dorset)

4.2. Patients

4.2.1. Gene rearrangements in tumour and presentation blood

In order to identify tumour markers, gene rearrangement studies were performed on tumour samples from 18 patients with AML (FAB M_1 7, M_2 2, M_3 3, M_4 3, M_5 3; 9 male, 8 female; mean age 46.4 years, range 14 - 73), 6 patients with ALL (5 male, 1 female, mean age 22.5 years, range 16 - 30), 12 patients with NHL (6 high grade, 6 low grade, mean age 44.1 years, range 15 - 63) and 9 patients with HD (mean age 33.4 years, range 14 - 51).

Gene rearrangement studies were used to assess the presence of tumour in the peripheral blood of 36 patients with NHL at presentation. Seventeen patients had high grade B cell NHL (mean age 60.2 years, range 41 - 80), 3 patients had high grade T cell NHL (mean age 48 years range 39 - 57) and 16 patients had low grade B cell NHL. Six patients had stage I disease, 2 stage II, 8 stage III and 21 stage IV. Ten patients had bone marrow involvement.

AML was classified according to the FAB criteria (Bennett et al, 1985}, ALL according to MIC criteria (First MIC Cooperative Study Group, 1986), and NHL according to the Keil classification (Stansfield et al, 1988) Patients with lymphoma were clinically staged according to the Ann Arbor system (Carbone et al, 1971).

Peripheral blood counts were performed as previously described (2.3.5) and May Grunwald Giemsa stained films examined by light microscopy for the presence of tumour cells. Bone marrow involvement was assessed by light microscopic examination of bone marrow aspirate and trephine biopsies.

4.2.2. PBSCH

Tumour contamination in 60 PBSCH from 22 patients was investigated by gene rearrangement studies (Table 4.I). Of the 7 patients with NHL, 6 were stage IV and one stage I. Similarly. one patient with HD was stage I, one stage III and 5 stage IV. Two patients with AML were FAB M_1 and one M_5. Three patients had common ALL and one B ALL.

Disease	n	mean age (range y)	M/F	lineage
AML	3	26.3 (17-32)	1M/2F	
ALL	4	23.3 (17-30)	3M	1 B ALL 3 cALL
NHL (High grade)	4	41.5 (24-55)	2M/2F	1 B cell 3 T cell
NHL (Low grade)	3	42 (41-44)	2M/1F	3 B cell
HD	7	31.6 (14-57)	5M/2F	
Myeloma	1	61	1M	

TABLE 4.I Characteristics of patients whose PBSCH were assessed for tumour contamination by gene rearrangement studies.

4.3. Methods

Methods were based on those of Maniatis (Maniatis et al, 1982).

4.3.1. Isolation of DNA

4.3.1.1. Isolation of DNA from blood and bone marrow

Five volumes of red cell lysis buffer at 37°C was added to one volume of blood or bone marrow and incubated at 37°C for 5 min to lyse the red blood cells. The sample was spun at 700g for 10 min and the supernatant removed. The remaining cell pellet was washed twice in 0.9% saline and resuspended in high TE. The cells were lysed by adding an equal volume of lysis solution. RNAse A was added at a final concentration of 50 ug/ml, and incubated at 37°C for 30 min. Proteinase K was added at a final concentration of 100 ug/ml and incubated at 40°C for 16 h.

The sample was extracted twice in one volume of Tris buffered phenol followed by one volume chloroform-isoamyl alcohol (24:1). The DNA was precipitated by adding 0.5 volume 7.5M ammonium acetate and 2 volumes of absolute ethanol. The long threads of DNA were spooled on to a glass rod, washed with ammonium acetate/ethanol (1:2) then washed with absolute ethanol and allowed to dry until tacky. The DNA was resuspended in 500μl TE and

allowed to dissolve at 4°C for at least one week before use. Dissolved DNA samples were stored at -70°C.
If the DNA concentration was so low that no threads were seen after the addition of ethanol, the samples were placed at -40°C overnight, centrifuged at 1600g for 30 min at 4°C . The DNA pellet was resuspended in 1 ml of 70% ethanol, transferred to an mcc tube and centrifuged at 16000g for 10 min at 4°C. The DNA pellet was dried under vacuum, resuspended in 100µl TE and stored at 4°C. The concentration and purity of the sample were estimated using UV spectroscopy. Samples were diluted 1:10 with DW and the absorbance measured at 260 and 280nm. The concentration of DNA was calculated using the ratio of 1.0 OD_{260} equivalent to 50µg DNA. The purity of the DNA was assessed using the ratio OD_{260} : OD_{280}.

4.3.1.2. Isolation of DNA from mononuclear cells
MNC, isolated from blood, marrow or PBSC harvest by Ficoll-hypaque density gradient centrifugation were collected in IMDM + 2% FCS. These were spun at 700g for 5 min and the medium discarded. The cells were resuspended in one volume of high TE to which one volume of lysis solution was added. The lysate was treated as outlined previously (4.3.1.1.).

4.3.1.3. Extraction of DNA from tissue samples
Fresh tissue (eg lymph node, spleen) was macerated with a razor blade as finely as possible, suspended in high TE and an equal volume of lysis buffer added. The lysate was

treated as outlined previously (4.3.1.1.).

4.3.2. Digestion of DNA by restriction endonucleases

To 7μg DNA, restriction endonuclease (EcoRI, HindIII, BamHI) at a ratio of 10u/μg DNA, an appropriate volume of 10x concentrated reaction buffer and DW was added to a final volume of 70 μl. The digest was incubated at the appropriate temperature for the restriction endonuclease for 18 h. The reaction was stopped by adding 12 μl of gel loading buffer.

4.3.3. Separation of DNA fragments by gel electrophoresis

Digested DNA samples were loaded onto a 0.8% agarose gel in TAE and electrophoresed for approximately 18 h at 50 V (0.3 - 0.4A) until the bromophenol blue marker dye had travelled 18cm from the wells. The gel was stained with 0.05% ethidium bromide in TAE for 15 min, destained in DW for 5 min and photographed under UV light.

4.3.4. Southern blotting

The gel was placed in several volumes of acid hydrolysis solution to break covalent bonds and shear DNA into pieces and gently shaken for 30 min with one solution change. The gel was then transferred to denaturing solution for 45 min with 2 solution changes, to denature the double-stranded DNA to single-stranded fragments. The DNA fragments within the gel were blotted onto a

nitrocellulose filter by capillary blotting. The gel was placed on top of a wick of filter paper (2 sheets Whatman No.6, 3mm paper) supported on a glass plate, with the ends of the wick submerged in 20x SSC solution. A nitrocellulose filter, cut to the same size as the gel and soaked in 2x SSC, was placed carefully on top of the gel. Above this was placed 3 sheets of filter paper (Whatman No.6) cut just larger than the size of the gel, a 15 cm pile of paper towels, a glass plate and a brick. Capillary blotting occurred over 18-24 h, changing the saturated paper towels when necessary.

The filter was retrieved, marked and washed in 2 x SSC and baked for 2 h at 80°C. The filters were stored at room temperature.

4.3.5. Probes

The probes used were a 2.5 kb EcoR1 - BgIII fragment containing the IgHJ region (Flannagan et al, 1982), 0.8 kb SacI - XhoI fragment containing the TCR Cβ1 gene (Gledhill et al, 1990) and a 1.8 kb SacI fragment of the region 3' to the TCRJδ1 (JδS16 probe) (Boehm et al, 1988). DNA probes were derived from the plasmids by cleavage with an appropriate restriction endonuclease, electrophoresed in low melting temperature agarose, and the appropriate DNA fragments to be used as hybridisation probes cut out of the gel. The probes were extracted once in phenol/chloroform/isoamyl alcohol (25:24:1) and precipitated for 60 min at -20^{o}C after addition of 0.1 volumes of 3M sodium acetate (pH 5.2) and 2 volumes of

absolute ethanol.

The probes IgHJ, TCRβ and TCRδ were radioactively labelled by the random primer oligolabelling method based on that of Feinberg and Vogelstein (1983 & 1984) to a specific activity of not less than 8 x 10^8cpm/μg DNA. To a volume containing 25ng of linearised probe DNA was added 3 ul 0.5M nucleotide (dATP, dGTP and dTTP) solution, 2ul hexanucleotide and buffer solution, 2U Klenow enzyme and 5ul ^{32}P-labelled d-CTP (50 uCi) This was incubated at room temperature overnight. To ascertain incorporation of radioactivity, a spot of the reaction mixture was placed on GF/B filter and this was transferred to 10ml 5% TCA in a scintillation vial and the dpm measured on a β counter. The filter was retrieved, incorporated radioactivity precipitated with 5% TCA and the dpm counted. The incorporation was expressed as a percentage of the initial count. The sample was passed down a sephadex column to remove unincorporated nucleotides, and the specific activity of a 2 ul sample in 5 ml of scintillant was measured using a scintillation counter. The probes were used immediately.

4.3.6. Prehybridisation, hybridisation and washing

4.3.6.1. Prehybridisation

Twenty ml of prehybridisation solution and 2.5 mg sonicated salmon sperm DNA (heated to 100^{o}C for 10 min

and snap-cooled on ice to denature the DNA) was added to a polythene bag (Hybaid system) containing the filter to be probed and incubated at 65°C for at least 5 h.

4.3.6.2. Hybridisation

The prehybridisation solution was drained from the bag and to this was added the radiolabelled probes, plus a further 2.5 mg sonicated denatured salmon sperm DNA. This mixture was returned to the bag, inverted several times to ensure uniform bathing of the filter and the air removed. The filter was incubated at 65°C for 16 h.

4.3.6.3. Washing

Using the Hybaid apparatus the filter was washed with wash solution 1 for 5 min at room temperature and the radioactivity remaining in the bag monitored with a hand held Geiger counter. Depending upon the probe used and the monitored counts, wash solution 2 and then wash solution 3 were used for 5 min at 65°C until the counter registered <2 cps.

4.3.7. Autoradiography

The filter was wrapped in cling film and mounted in an X-ray cassette next to an X-ray photographic film and left at -70°C. The film was developed in developer solution for 5 min, transferred to a stop bath for 30 seconds and fixed in fixative for at least 2 min before being washed in tap water for 30 min and dried.

4.3.8. Re-probing of a filter

The probe was removed by immersing the filter in probe stripping solution for 10 min, washing in 2x SSC and then exposing the filter to an X-ray film to check completeness of stripping.

4.4. RESULTS

4.4.1. Gene rearrangements in tumour samples

4.4.1.1. Acute leukaemias

Of the 18 patients with AML, 3 (17%) had rearranged bands when studied with TCRδ. An example of an autoradiograph probed with TCRδ is shown in fig 4.1. These patients had AML of different FAB types (1 M_1, 1M_2 and 1M_4) and only one was Tdt positive. Three other patients with Tdt positive AML (2M_1, 1M_{5a}) demonstrated no TCRδ rearrangements.

The 6 patients with ALL had disease of the B cell lineage and all demonstrated rearranged bands when probed with IgHJ. An autoradiograph of DNA from a patient with ALL probed with IgHJ is shown in fig 4.2. Four of the patients were also studied with TCRδ and all showed rearrangements (Table 4.1).

4.4.1.2. Lymphomas

All 8 patients with B cell NHL (2 high grade, 6 low

grade) had IgHJ rearrangements in their tumour. Three of the 4 patients with high grade T cell NHL had TCRβ rearrangements but one with large cell anaplastic NHL showed only germline configuration when probed with IgHJ, TCRβ and TCRδ. No tumour marker bands were found in any of the 9 patients with HD probed with TCRδ or 5 probed with IgHJ. (Table 4.II)

	PROBES		
	IgHJ	**TCRβ**	**TCRδ**
AML	0/3	n/d	3/18 (17%)
ALL	6/6	n/d	4/4
B NHL high	2/2	0/1	n/d
T NHL high	0/4	3/4 (75%)	0/3
B NHL low	6/6	n/d	n/d
HD	0/5	n/d	0/9
	14/26	3/5	7/34

TABLE 4.II Gene rearrangements in tumours from patients with haematological malignancies.

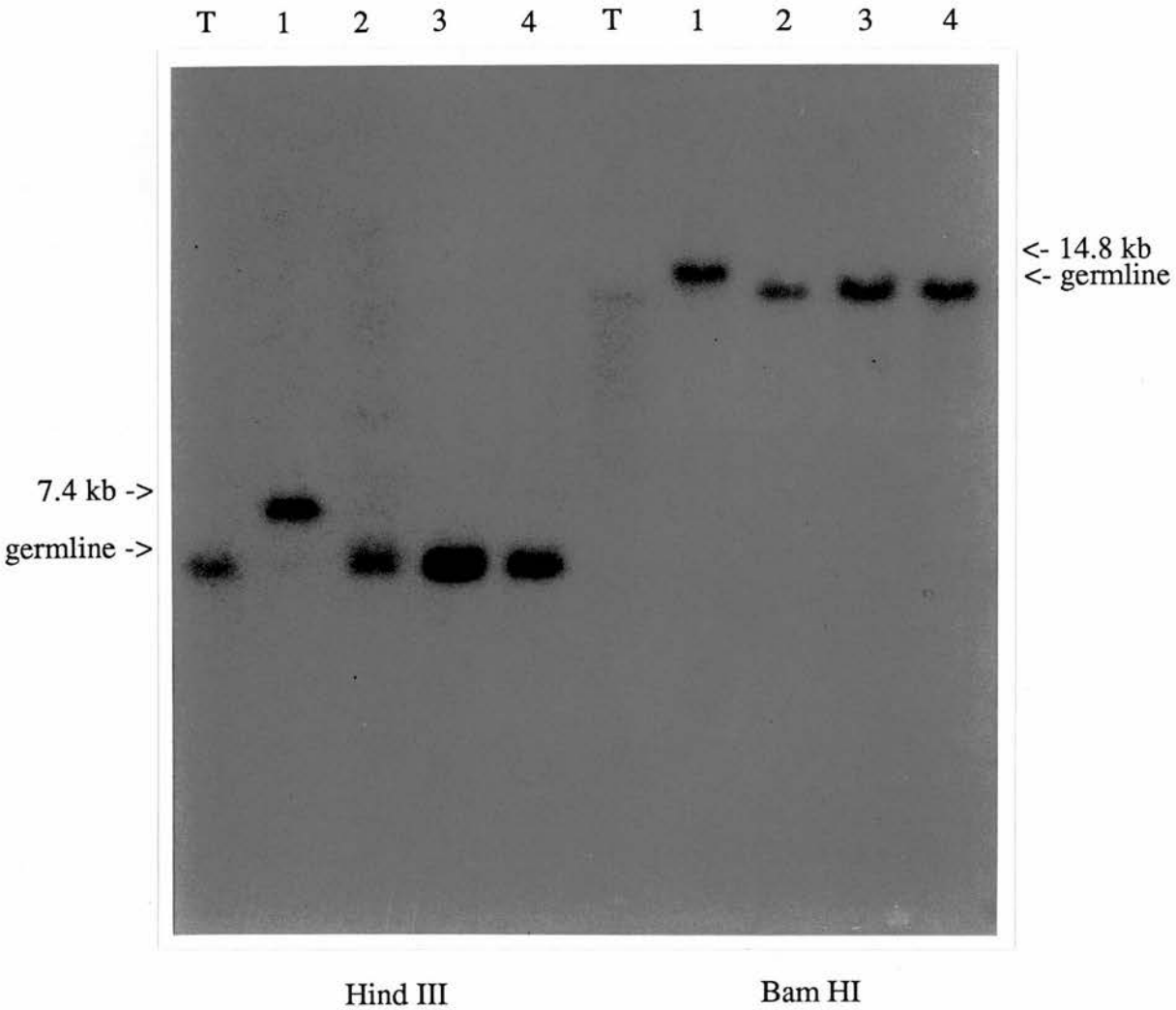

FIGURE 4.I Autoradiograph of DNA from 4 patients with AML probed with TCRδ. Tonsil DNA (T) was used to indicate germline configuration. A tumour marker band was demonstrated in patient 1. This measured 7.4kb after the DNA was digested with Hind III and 14.8kb after digestion with Bam HI. Patients 2,3 and 4 produced only germline bands.

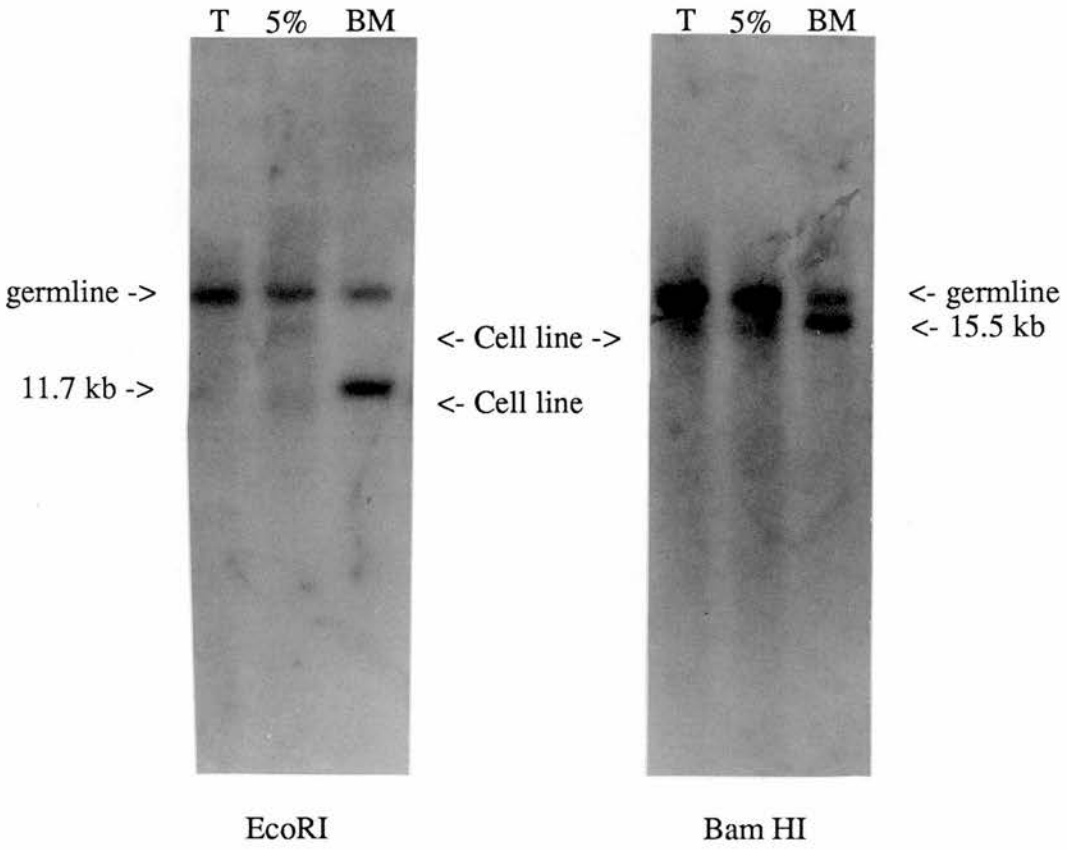

FIGURE 4.2 Autoradiograph of DNA from a patient with ALL probed with IgHJ. Tonsil DNA (T) was used to indicate germline configuration. A tumour marker band was demonstrated in the patient (BM). This measured 11.7kb after the DNA was digested with EcoRI and 15.5kb after digestion with Bam HI. The sensitivity of the technique was determined by dilution of a cell line with an IgHJ rearrangement in normal DNA. A cell line marker band was demonstrated at a concentration of 5% confirming the level of sensitivity.

4.4.2. Gene rearrangements in presentation blood in patients with NHL

As 8 of 8 patients with B cell and 3 of 4 patients with T cell NHL had tumour marker bands presentation blood samples were assessed for tumour involvement using IgHJ and TCRβ gene probes. Of the 36 patients studied, 7 (19.4%) had rearrangements detectable in their peripheral blood. This included 3 of 20 (15%) patients with high grade disease (3/17 B cell, 0/3 T cell) and 4 of 16 (25%) with low grade disease (all B cell). Conventional microscopic examination of blood films detected tumour cells in only 2 of these 7 patients.

Peripheral blood gene rearrangements were more often found in stage III/IV disease (21%) than in stage I/II (12.5%) and occurred in 40% of those with bone marrow involvement. Despite the bone marrow appearing histologically clear of disease in 26 patients, 3 (1/20 high grades and 2/16 low grades) had evidence of gene rearrangements in the blood (11.5%).

Bone marrow gene rearrangements studies were performed in 24 patients (12 high grade, 12 low grade) and marrow involvement found in 7 (29%). In 4 (57%) of these patients examination of bone marrow trephine and marrow aspirate had failed to demonstrate malignant cells. In one patient, marrow involvement found on microscopic examination failed to be detected by DNA examination.

4.4.3. Gene rearrangements in PBSCH

The results of Southern blotting analysis of PBSCH in patients with AML and HD are shown in table 4.III. As tumour markers were not found with any probe, the PBSCH were unable to be assessed for tumour contamination by gene rearrangement analysis.

All patients with ALL, 3 of 4 patients with high grade NHL and all patients with low grade NHL had tumour markers demonstrable by gene rearrangement analysis. An identical rearrangement to the tumour was found in the presentation bone marrow of 3 patients and in the peripheral blood of 1 patient with NHL. No rearrangement was found in any of the PBSC harvests using this technique (Table 4.IV). The results of gene rearrangement studies from 2 patients with NHL are shown in figs 4.3 and 4.4.

Disease	Patient	Probe	Tumour	PBSC(n)
AML	FC	TCRδ	G	G(3)
	KD	TCRδ	G	G(2)
	SW	TCRδ	G	G(2)
HD	DB	TCRδ	G	G(2)
		IgHJ	G	G
	GM	TCRδ	G	G(2)
		IgHJ	G	
	EN	TCRδ	G	G(1)
		IgHJ	G	G
	DO	TCRδ	G	G(3)
		IgHJ	G	G
	BP	TCRδ	G	G(2)
		IgHJ	G	G
		TCRβ	G	G
	AS	TCRδ	G	G(5)
		IgHJ	G	G
	LT	TCRδ	G	G(3)
		IgHJ	G	G

TABLE 4.III Gene rearrangement studies on PBSCH from patients with AML and HD.

Disease	Patient	Probe	Tumour	PBSC(n)
ALL	GG	IgHJ	**R**	G(1)
		TCRδ	**R**	G
	WL	IgHJ	**R**	G(3)
		TCRδ	**R**	G
	BM	IgHJ	**R**	G(1)
		TCRδ	**R**	G
	DP	IgHJ	**R**	G
		TCRδ	**R**	G
NHL	RB	IgHJ	**R**	G(2)
(high grade)		TCRβ	G	G
		TCRδ	G	G
	MA	IgHJ	G	G(2)
		TCRβ	G	G
		TCRδ	G	G
	TH	TCRβ	**R**	G(4)
		TCRδ	G	G
	MP	IgHJ	G	G(5)
		TCRβ	**R**	G
		TCRδ	G	G
NHL	AM	IgHJ	**R**	G(3)
(low grade)	SP	IgHJ	**R**	G(6)
	LW	IgHJ	**R**	G(2)
Myeloma	JB	IgHJ	**R**	G(1)

TABLE 4.IV Gene rearrangement studies on PBSCH from patients with ALL, NHL and myeloma

4.5. Conclusions

Gene rearrangement studies identified tumour markers in all patients with ALL, 17% of AMLs, 92% of NHLs and in no patients with HD. Of the patients with NHL, 19.4% demonstrated gene rearrangements in the blood at presentation which was more often found in patients with advanced disease and bone marrow involvement. Light microscopy failed to demonstrate blood contamination in 70% of these cases and in 58% of patients with gene rearrangements detected in marrow samples. Because of the lack of tumour markers for AML and HD gene rearrangement studies were useful for assessing tumour contamination in only 60% of PBSCH, but in these no evidence of disease was found.

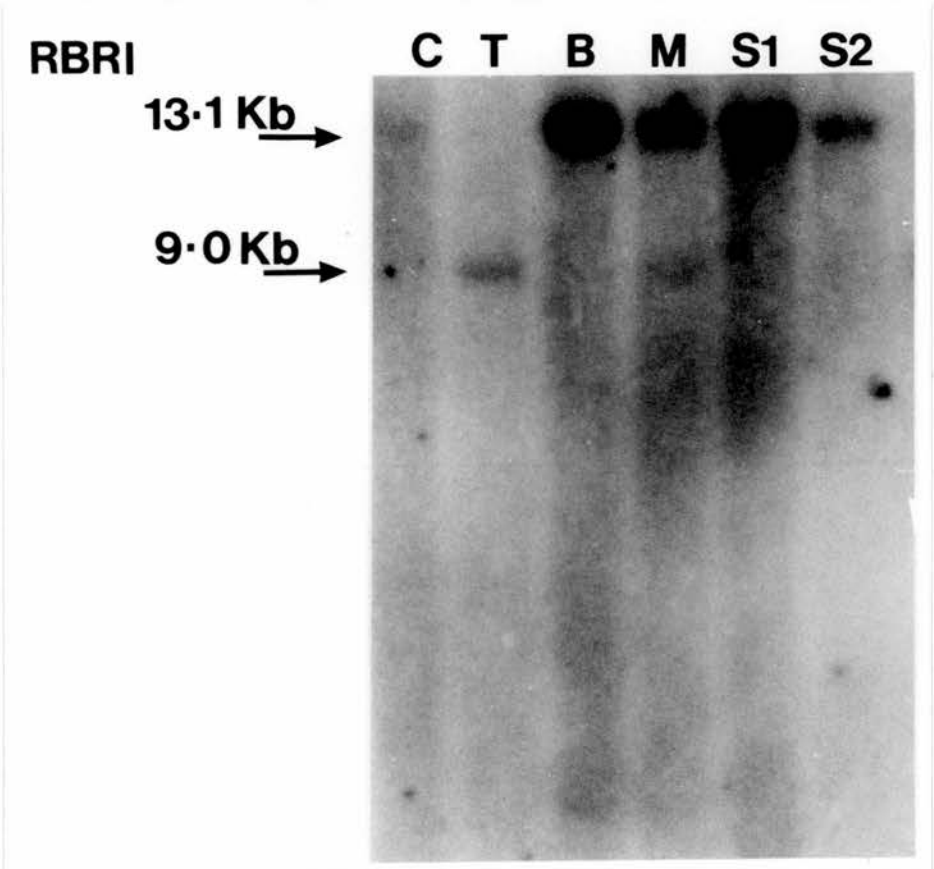

FIGURE 4.3 Autoradiograph of DNA digested with EcoR1 from a patient with high grade B cell NHL probed with IgHJ. Tonsil DNA (C) was used to indicate the 13.1kb germline band. A tumour marker band measuring 9.0kb was demonstrated in tumour (T) and marrow (M) but not in blood (B) or 2 PBSCH (S1 and S2).

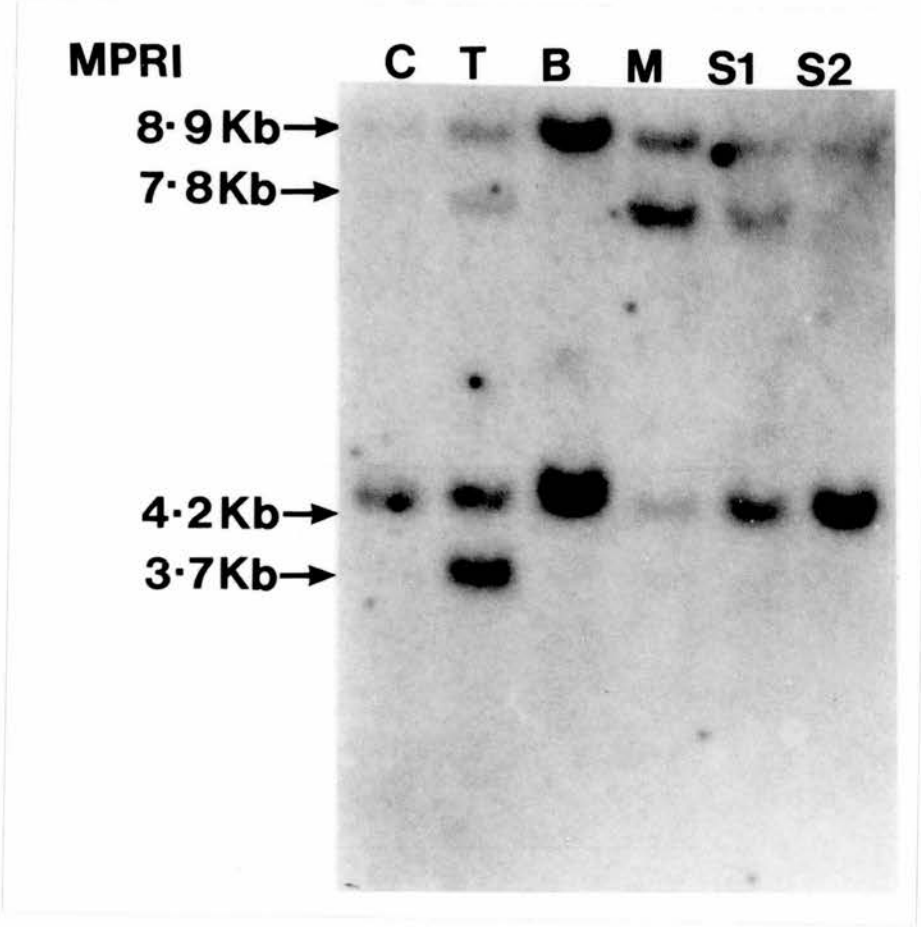

FIGURE 4.4 Autoradiograph of DNA digested with EcoR1 from a patient with high grade T cell NHL probed with TCRβ. Tonsil DNA (C) was used to indicate the 8.9kb, 7.8kb and 4.2kb germline bands. A tumour marker band measuring 3.7kb was demonstrated in tumour (T) and marrow (M) but not in the blood or 2 PBSCH (S1 and S2).

CHAPTER 5

DISCUSSION

In order to increase the availability of autologous transplantation for patients with haematological malignancies, blood was investigated as an alternative source of stem cells. The response of PBSC was characterised after standard chemotherapy for haematological malignancies and the feasibility of harvesting these progenitors established. The harvest obtained was assessed for tumour contamination. These studies will now be discussed.

5.1. The CFU-GM assay

The ability of transplanted marrow to reconstitute and sustain haematopoiesis can be predicted not only by the measurement of CFU-GM (Spitzer et al, 1980) but also by the numbers of MNC infused (Gorin, 1986). As marrow MNC are considerably easier to measure than CFU-GM and give reproducible results from centre to centre they are used more frequently as a guide to dose.

The situation is quite different in the peripheral blood. Because of the flux of PBSC numbers in response to factors such as chemotherapy, the numbers of MNC do not correlate with CFU-GM or reconstitutive ability, and thus

are of little or no value. Progenitor cell culture assays are therefore of paramount importance as a guide to stem cells in the peripheral blood.

5.1.1. Standardisation of the CFU-GM assay system

The basic CFU-GM assay has several inherent problems that lead to variable reproducibility between centres and even within institutions. The assay is not standardised; the support material may be methylcellulose, as used in this study, or agar; FCS has variable growth promoting activity and this must be checked prior to batch changes; conditioned medium can be obtained from peripheral blood lymphocytes, placenta or from cell lines; colony counting shows inter and intra-observer variation.

In an attempt to minimise this variability, the following steps were taken:

1. The accurate identification of colonies was initially established by comparing photographs of the colony in situ on the plate with photographs taken after the colony had been removed and stained. Thus identification of colonies containing only one lineage of cell could be distinguished from those of mixed lineage and this operator skill could be checked at intervals.

2. In peripheral blood the number of accessory cells present has been reported to produce an inhibitory effect on CFU-GM at high plating concentrations (To et al, 1983) and result in a non-linear plating concentration effect. In the assay system used in this study, there was no obvious inhibition of growth, a linear relationship

between MNC plating numbers from 0.5 - 8 x 10^8 and colonies per plate was observed for both normals and stem cell harvests. The optimal plating concentration was chosen as 2 x 10^5MNC/plate without monocyte depletion.

3. To standardise the conditioned medium, peripheral blood lymphocytes derived from one healthy donor after a period of exercise were used.

4. In order to monitor variability in the assay with time, to establish a normal range for our laboratory and to compare results with those from other centres, circulating CFU-GM from normal volunteers was assessed throughout the 3 year study period. No significant difference was found between normal results from different periods ($p = 0.38$) confirming the assay was consistent.

The mean value of 166 ± 19 CFU-GM/ml blood in the normal group is comparable to other normal values reported. A similar methylcellulose culture system with PHA LCM but a plating concentration of 1 x 10^6 MNC per plate was used by a group in Italy who found the mean CFU-GM growth in 18 normals to be 251 ± 53 (1 SEM) (Carlo-Stella et al, 1987). A similar system was employed in Canada, plating at 3 x 10^5MNC where 12 normal samples had a mean value of 60 CFU-GM per ml, range 7 - 168 (Cantin et al, 1989). Lasky et al, 1982 reported an even lower mean at 40 CFU-GM/ml. These figures demonstrate the amount of inter-laboratory variation despite similar culture systems.

Other workers using methylcellulose and feeder layers

have found ranges of 20 - 150 CFU-GM/ml (Partanen et al, 1982), 40 - 120 CFU-GM/ml (Chervenick, 1973) and 6 - 200 CFU-GM/ml (Hibben et al, 1984), whilst those working with agar systems have reported normal CFU-GM values of 149/ml (range 31 - 362) (Korbling et al, 1980) and 110/ml (95% confidence intervals 23 - 490) (To et al, 1989).
In an attempt to circumvent the interlaboratory variation in normal values the peak values obtained after chemotherapy are compared to those from normals and expressed as a proportional increase above the mean.

5.1.2. Future improvements in PBSC identification
Despite meticulous aseptic technique, sterilisation procedures and multiple antibiotics, bacterial and fungal infection occurred periodically, especially when any repair or reconstruction work was being done in the vicinity of the department. The 2 week incubation period was a major drawback to the system as results were not available quickly to aid clinical decision making. However, despite its disadvantages, the CFU-GM assay at the time represented the best experimental assessment of haematopoietic progenitor cells as it is the most studied and most widely accepted predictor of haematopoietic reconstitutive capacity.

Many of the disadavantages of the progenitor cell assay system may be overcome by the use of immunophenotyping. Recently it was demonstrated that CD 34+ cells in the

circulation detected by dual colour direct immunofluorescence flow cytometry after PBSC mobilisation correlated well with CFU-GM (Serke et al, 1991; Siena et al, 1991). Subpopulations of these CD34+ cells were found to express CD33 and these correlated with early haematopoietic recovery of marrow function, predicting this more accurately than total MNC or CFU-GM infused (Siena et al, 1991). If confirmed, this method will provide rapid, accurate and reproducible guidelines for estimation of peripheral blood stem cells.

5.2. PBSC after chemotherapy

The continued debate over the minimum numbers of circulating CFU-GM required to sustain engraftment in various disorders highlights its lack of reliability as a predictor of engraftment. Until a more reliable predictor is found, the aim of PBSC collection must be to collect as many progenitor cells as possible. This may be maximised if PBSC harvests are performed when the stem cell pool is expanded as, for example, on recovery from chemotherapy. The most studied group of patients are those with AML where chemotherapy has produced an 11 - 25 fold increase in CFU-GM in early remission (To et al, 1984; Giessler et al, 1986) which have been successfully transplanted. In patients with solid tumours a 14 fold rise in CFU-GM was demonstrated after chemotherapy for small cell carcinoma of lung (Stiff et al, 1987), a 20

fold increase after treatment for ovarian cancer (Richman et al, 1976) and a 10 fold increase after IMVP16 for lymphoma in 2 patients (Bell et al, 1987b). Recently the introduction of high dose schedules such as cyclophosphamide 4g/m^2 to specifically induce a rise in PBSC has been reported. A mean 14 fold rise predictably occurred in 69% of patients, but with significant morbidity and even mortality (To et al, 1990). The precise response to standard chemotherapy regimes, however, such as CHOP, ALL, ChlVPP, MOPP, for patients with lymphomas has not been reported and these may represent a less toxic way of inducing a stem cell increment.

5.2.1. PBSC after treatment for acute leukaemias.

In AML, this study demonstrated a 38 fold increase in CFU-GM over normal values after DAT chemotherapy occurring on average at day 28.6 after the start of treatment, and a 12.5 fold increase after ADE at day 26. No rise in circulating progenitors was found in 2 patients neither of whom entered remission after the first course of treatment. An extremely large rise in PBSC was found in one patient with M_3 AML after DAT 1 (34,243/ml), however, he was not in remission and also had a high blood CFU-GM prior to treatment. It seems likely that these PBSC may be contaminated with the malignant clone.

Patients with ALL also demonstrated increased CFU-GM to 5.3 fold after induction therapy at day 24 and a larger

increase after intensification to 35 fold at day 26. All demonstrated an increase in CFU-GM, including one patient who did not enter remission although his results are the lowest for both induction and intensification.

These reults are in general agreement with other reports where mean increases ranging from 5.6 to 25 fold have been reported after DAT type therapy for AML (To et al, 1984; Cantin et al, 1989). PBSC increases reported here with DAT are considerably higher than those reported by Reid et al despite a similar chemotherapy regime. However, Reid quoted no normal values for his laboratory limiting direct comparisons with my results, additionally the numbers of patients studied were small. Only 3 of Reid's patients were receiving first induction treatment and it was emphasised that circulating CFU-GM fell markedly with repeated courses of treatment (Reid et al, 1989). The differences in the levels of CFU-GM after chemotherapy in the present study, as shown by the large ranges (Table 2.VIII), stresses the variability of PBSC peaks and the difficulty in predicting when to collect PBSC.

In contrast, the levels of progenitors found after intensification therapy for ALL were very similar to those demonstrated by Reid and occurred at consistent times.

A factor associated in the present study with a high CFU-GM peak after chemotherapy was a rapid recovery from

myelosuppression, as indicated by the the time taken to increase the total white cell count from 1 to 3 x 10^9/l. Others have used the rapid recovery of monocytes or platelets to monitor this (To et al, 1990; Cantin et al, 1989; Reid et al, 1989). Two successive courses of chemotherapy did not significantly reduce the mean peak of the group as a whole, however the numbers were small and variations in response were seen when patients were considered individually (Fig 2.4). There was no evidence, however, of the major reduction in peak values after these 2 courses, as found by Reid et al, our results being more in keeping with those of Cantin et al, 1989 who found no relationship between the levels of CFU-GM and the number of DAT cycles administered.

Thus, for patients with leukaemia it was considered that to maximise PBSC harvested, leukapheresis should start in first remission, at times determined by the specific regime. This should occur usually after the total WCC increased to greater than 1 x 10^9/l and as counts were rapidly rising over a period of 5 days. The leukapheresis should be repeated after at least the second course of chemotherapy.

5.2.2. PBSC after treatment for lymphoma

In patients with lymphoma, sampling was performed less frequently than in patients with leukaemia. The major reason was patients with lymphoma were predominantly treated as out-patients whereas those with acute

leukaemia stayed in hospital throughout the period of myelosupression which was more severe and prolonged. Because of a lower sampling frequency, it is possible that CFU-GM peaks were wholly or partially missed, and accurate predictors of recovery such as those studied in patients with acute leukaemia, could not be fully assessed.

Despite these limitations, standard chemotherapy regimes for lymphoma induced a documented rise in circulating CFU-GM in 87% (40/46) of patients. In those patients with high grade NHL, the increase found after ALL induction therapy (17.5 fold) was higher than after induction therapy in patients with ALL (5.3). The increase occurred a little earlier and may reflect the lack of marrow infiltration by malignant cells in the patients with NHL. Patients treated with CHOP based regimes demonstrated a 6.9 fold increase in CFU-GM, with only one patient having no documented rise, PEEC gave very large increases to 48 fold. These levels of PBSC are comparable to those found in other patients with lymphoma (Bell et al,1987b), solid tumours (Richman et al, 1976; Stiff et al, 1987) and acute leukaemia (To et al, 1987a) and therefore should yield reasonable numbers of PBSC for transplantation. The timing of progenitor peaks for out-patients was generally towards the end of the third week of the chemotherapy cycle as counts were recovering and monocytes were elevated (mean 12.5%).

Less intensive regimes for low grade disease produced

only minor increases in CFU-GM and therefore patients were not considered for PBSC harvesting.

Standard chemotherapy for HD resulted in a less frequent mobilisation of PBSC. This may have reflected the patient population, as 6 had undergone previous chemotherapy regimes and 3 had bone marrow involvement both of which produced significantly lower CFU-GM peaks in the lymphoma group as a whole (figs 2.9 & 2.10). The more prolonged type of chemotherapy regime or the inclusion of chlorambucil which damages early stem cells, eg ChlVPP, OPEC, may also reduce stem cell mobilisation. Large increases were seen after HOPE which contained 40mg/m^2 of adriamycin and produced considerable myelosuppression with subsequent CFU-GM rebound.

Thus, standard chemotherapy for lymphoma can induce a rise in PBSC in the majority of patients with lymphoma. Higher peaks are found in those receiving first line chemotherapy and in those without marrow involvement. As previous chemotherapy diminishes the response of CFU-GM, in those patients with a high risk of relapse, harvesting of progenitors should be performed during first line chemotherapy after the first course of treatment as white cell counts are recovering and should be repeated after each cycle.

5.2.3. Myeloma

A group of patients with myeloma were studied, but results were inconclusive. Only a small number of

patients were studied, 75% of whom had received previous treatment and all of whom had diseased marrow. PBSC increases were poor. In such patients, high dose chemotherapy such as cyclophosphamide $7g/m^2$, is required to mobilise sufficient PBSC for autografting (Marit et al, 1990; Bell et al, 1990).

5.3. Other approaches to PBSC mobilisation.

Apart from standard chemotherapy for malignancies, PBSC have been mobilised using high doses of chemotherapy, in particular cyclophosphamide (Marit et al, 1990; To et al, 1990). This approach, which has significant side effects, increased stem cells in 69% of cases but was less successful in those with over 20% marrow infiltration and previous myelotoxic chemotherapy which may constitute a significant number of patients who could potentially benefit from PBSCT.

The recent cloning of various haematopoietic growth factors has led to their production in sufficient quantity and purity for clinical study. G-CSF stimulates the production and function of granulocytes and shortens the period of neutropenia after chemotherapy and bone marrow transplantation (Gabrilove et al, 1988; Teshima et al, 1989). Dexter's group demonstrated the presence of substantial numbers of primitive stem cells in the peripheral blood of mice after G-CSF treatment, which were capable of reconstituting the haematopoietic system

in the long term and of contributing to the lymphoid populations of the thymus when transplanted into irradiated mice (Molineux et al, 1990). In man, evidence exists that the less lineage specific growth factor GM-CSF has also shortened the period of neutropenia after BMT (Brandt et al, 1988; Nemunaitis et al, 1988) but with more side effects than G-CSF.

GM-CSF was used in combination with $7g/m^2$ cyclophosphamide to mobilise CFU-GM. Together these 2 modes of treatment increased CFU-GM up to 1000 fold (Gianni et al, 1989). These progenitors were harvested and reinfused together with bone marrow after which, in some cases, further treatment with GM-CSF was given. Rapid haematopoietic reconstitution was observed, however the contribution made by the GM-CSF mobilised CFU-GM is unclear and their ability to sustain long term haematopoiesis was not established. Haas used GM-CSF alone to mobilise CFU-GM in patients with non-myeloid malignancies and found a mean increase of 8.5 fold after 10 days. These were harvested and reinfused to 6 patients after myeloablative treatment and restored adequate haematopoiesis in 5 with a follow up of a median of 103 days (Haas et al, 1990).

Thus, the use of growth factors to mobilise stem cells appears promising but their ability to maintain long-term haematopoiesis in man has yet to be established. An interesting approach will be the use of G-CSF after standard chemotherpy to both shorten the periods of

neutropenia and possibly increase circulating PBSC in particular in those heavily pretreated individuals or those with marrow infiltration.

5.4 PBSC Harvests

Having established the occurrence and timing of CFU-GM increases after standard chemotherapy regimes for lymphoma and leukaemias, PBSC were harvested using a Fenwall CS3000 continuous flow cell separator. This machine offered operator independent consistency, being fully automated under computer control. As stem cells were found in the lymphocyte fraction of blood programme 3 (lymphocyte collection) was selected.

5.4.1. Clinical problems associated with PBSCH

During the initial assessment period 7 litres of blood were processed, but this was increased to a target volume of 9 litres. Five litres were processed in a boy of 14 who weighed only 33 kg and in one patient the procedure was terminated after 2.3 litres due to severe venous spasm. Not surprisingly, obtaining adequate venous access was difficult in some of those patients who had received irritant chemotherapy. The majority of such patients had long term indwelling central venous catheters inserted for drug administration and venous sampling. Adequate venous out-flow rates could be achieved through a Raaf double lumen catherter (Quinton, Kimal scientific

products ltd, Uxbridge) internal dimensions 1.2 x 3mm. The return line could then be attached to a smaller 16G cannula. If an adequate out flow line could be established peripherally then the return line could be connected to a central line of various diameters (Quinton triple lumen catheters internal dimension 1.0mm, double lumen catheters internal dimension 1.5mm). The patients found this a more comfortable arrangement as it allowed one arm free for drinking, reading, etc.

Two patients who had vasovagal attacks both recovered fully and were able to continue with the procedure and subsequent harvests. One gentleman became very anxious anticipating the procedure and the other omitted breakfast after a busy night of socialising. No other major complications (in particular severe headache as reported in patients with intracranial pathology on intermittent flow leucapheresis (Smith et al, 1990)) were encountered. Apart from the comment that the harvests could be rather tedious, being confined to a bed or chair for 3 hours, and the minor discomfort of cannulae insertion, the patients tolerated the procedure well; about half being performed as out-patients. No-one complained of paraesthesia due to hypocalcaemia from citrate toxicity as has been reported (To et al, 1989) although we have seen this recently in further studies with the Cobe Spectra.

Despite a 41% reduction in the circulating platelet numbers after the harvest, there was no evidence of

clinical bleeding. However, if possible, harvests were deferred until platelets were greater than 35 x 10^9/l or a platelet transfusion was given at the end of the procedure.

5.4.2. Product specification

The CFU-GM collecting efficiency of 65.7% in the CS3000 is comparable to that in patients with AML (58 - 73%) (To et al, 1989), in normal volunteers (64%) (Lasky et al, 1982) and in patients with leukaemia and lymphoma (76%) (Vannier et al, 1989) all harvested on the CS3000. However, the 200ml product obtained from the CS3000 contained significant red cell contamination with a haematocrit of 17% and a relatively high number of platelets (mean 2.5 x 10^{11}) which resulted in a 41% reduction in circulating platelets . Similar reductions in platelets have been reported using the CS3000 (Lasky et al, 1982), or the Haemonetics V50 (Schouten et al, 1990).

5.4.3. Advances in PBSC harvesting

In order to obtain adequate numbers of PBSC for engraftment, several apheresis procedures must be performed and the products cryopreserved. At the time of transplant there is generally a larger volume of product, including DMSO and red cells, to be returned to the patient than during BMT. Toxicity such as tachypnoea and elevations in serum bilirubin and creatinine can result

from large volumes of DMSO and contaminating red cells (Kessinger et al, 1990). The ideal stem cell harvest product should, therefore, be in a small volume with minimal red blood cell, polymorph and platelet contamination.

In order to reduce the numbers of platelets removed during the harvest, modified protocols have been investigated. Williams et al used a modified programme 1 (platelet collection) on the CS3000 with the interface detector set at 100, the centrifuge speed dependent on the patients haematocrit and giving a final soft spin at the end of the procedure to return platelet rich plasma to the patient. This collected between 2 - 4 x 10^{11} platelets with a 5% loss of MNC (Williams et al, 1990). Other groups have reported a lower CFU-GM collecting efficiency with programme 1 (Iacone et al, 1990). Similar modifications were investigated on the Haemonetics V50 where adding a lymphocyte surge to the standard programme reduced platelet loss by 40% and also reduced red cell contamination. Similar numbers of CFU-GM were collected by both methods indicating there may be some benefit in this modification without compromising the final product. Modifications to harvesting technology are being developed for the CS3000 with the introduction of a small collection chamber and modified computer programme to reduce the volume of the product and red cell and platelet contamination. The COBE Spectra cell separator is currently being evaluated in this laboratory for its

ability to produce a similar product.

5.4.4. Secondary processing

In view of the product obtained from the CS3000 and the risks of red cell and DMSO reinfusion, the harvest was purified by secondary processing over ficoll using an IBM 2991 cell processor. The goals of volume reduction and red cell depletion were achieved, but a 28% loss of CFU-GM occurred and the procedure itself was very time consuming and expensive. The use of a cell processor however, had an advantage over manual techniques for cell separation as it used a closed system and thus reduced the risk of microorganism contamination. Law et al reported no bacterial or fungal contamination using a Haemonetics V50 to separate MNC from 30 donor units (Law et al, 1988).

In a letter, Duoay et al recommended ficoll purification of PBSC which produced superior CFU-GM recoveries (79%) after cryopreservation, compared with only a 19% CFU-GM recovery in those unseparated PBSC. He suggested that unseparated cells were more easily damaged during cryo-preservation (Duoay et al, 1989). Unfortunately the full methods were not detailed in his letter, but perhaps if the harvests were contaminated with granulocytes the cells would be more likely to clump after cryopreserva-tion. In contrast, Kessinger et al found that PBSC harvests separated over ficoll resulted in delayed en-graftment suggesting damage or loss during processing

but she did not comment upon CFU-GM recoveries (Kessinger et al, 1990). Thus the role of ficoll separation remains unclear but the present study found loss of CFU-GM during the procedure.

5.4.5. Pellet Viabilities

In this study, CFU-GM culture studies of pellets to monitor viability of stored PBSC harvests may not be an accurate reflection of growth in the bulk PBSCH. Vital dye exclusion (eg trypan blue) is inadequate for assessment of progenitor survival. This method depends upon the ability of viable cells to exclude dyes and will measure damage to many cell populations. There is disparity between dye exclusion and CFU-C survival after freezing (Gray and Robinson, 1973) suggesting that other nucleated cells are lost preferentially. Measurement of CFU-GM recovery on pellets compared to the original product after processing, showed no loss of viability until 12 months when the growth was reduced by 30%. However, the growth in pellets did not appear to correlate with the actual growth found in the stored bags of PBSC at the time of reinfusion to patients. This observation has been noted by other workers (Foures et al, 1990). The most likely explanation lies in the storage and handling of pellets which are otherwise prepared and frozen in exactly the same way as the PBSC harvest. Once frozen in liquid nitrogen the pellets are stored in separate racks from the harvests which are

removed at regular intervals from the liquid nitrogen tanks in order to find other pellets from marrow and PBSC harvests for assessment. As the pellets contain only 0.5 ml the cells are probably subjected to temperature variations resulting in cell death.

5.4.6. Progenitors Harvested

Wide variations in CFU-GM dose have been reported to produce haematopoietic reconstitution, but it would appear that in non-leukaemic disorders a lower dose may be sufficient. For patients with AML doses of 30 - 50 x 10^4/kg are capable of engraftment while in other disorders CFU-GM above 5 x 10^4/kg result in rapid reconstitution (Korbling and Martin, 1988).

Overall, the CFU-GM yields were limited by similar factors that affected the CFU-GM peaks after standard chemotherapy regimes.

5.4.6.1. PBSCH in patients with lymphoma

In the group of lymphoma patients as a whole, a mean of 0.99 x 10^8 MNC/kg per procedure containing a mean of 2.93 x 10^4CFU-GM/kg were obtained (Table 3.III). In patients with NHL, the highest numbers of CFU-GM were harvested from those treated with ALL chemotherapy which is in keeping with the higher circulating CFU-GM found after this regime in the initial study. Two patients (SL and SP) had only 1 procedure carried out because of venous access difficulties. In the other patients, despite relatively few proceedures (2 - 5) more than 5 x

10^5 CFU-GM/kg in total were obtained. Current policy is to perform if possible, at least 2 PBSCH after each pulse of chemotherapy.

In patients with HD lower yields were obtained. One (EN) had the procedure terminated after only 2.3 litres because of venospasm, resulting in a poor harvest. Two patients (BP and GL) had harvests resulting in good CFU-GM yield after only 2 or 3 passes. The other patients (except AS) were all receiving 2nd line or greater chemotherapy which reduced the amount of circulating CFU-GM and numbers harvested. Patient AS had bone marrow involvement which resulted in lower PBSC and in addition was receiving ABVD which only produced CFU-GM increments in 6/9 of our patients.

5.4.6.2. PBSCH in acute leukaemia

More CFU-GM were harvested from the group of patients (Table 3.IV) with leukaemia than those with lymphoma reflecting the higher CFU-GM peaks found after this form of chemotherapy. Relatively few CFU-GM were collected from GG because of mis-timing of the harvest and from KD who was receiving the second pulse of treatment for first relapse and thus was heavily pre-treated. FC, SW and JH were harvested after MACE and MidAc which are the third and fourth pulses of chemotherapy for AML in the MRC AMLX protocol. The response of PBSC after these chemotherapy regimes had not been included in this study, but others have shown that in AML, after 2 courses of treatment

there can be a fall in CFU-GM (Reid et al, 1989). In 3 patients who had well timed harvests after first or second courses of chemotherapy (DC, DP and WL) good CFU-GM yields were obtained.

Thus, PBSC harvesting after standard chemotherapy can be successful if performed with correct timing and after early courses of treatment in patients with acute leukaemia, NHL and some patients with HD, depending on the regime used. For those patients who have had previous chemotherapy or those with marrow involvement other means of stem cell mobilisation may have to be considered.

5.5 Gene rearrangements in haematological malignancies

Gene rearrangement studies may be a useful tool as a tumour marker in haematological malignancies, however there are several limitations to the technique. One of the most significant disadvantages is low sensitivity. Using dilution experiments with cell lines and normal DNA tumour at the level of 4% was consistently detected, the previously reported sensitivity level ranging from 1 to 10% (Cleary et al, 1984; Aisenberg et al, 1987). The detection of 1 malignant cell in 500 (0.002%) was never achieved, as claimed in ALL remission marrows (Zenbauer et al, 1986). The isolation of DNA, especially from solid tissue, is prone to sampling error if a malignant process is patchy in distribution and DNA is susceptible to degradation if there is delay in its extraction. This may

lead to false negative results. The multistage, complex nature of the DNA analysis leads to delay in obtaining results and offers scope for error and difficulties in interpreting the results. Despite these drawbacks gene rearrangement studies provided a tumour marker for a large number of haematological malignancies which could be used to assess tumour contamination.

5.5.1. Acute leukaemia

Tumour markers as evidenced by gene rearrangements were found in all 6 patients with B lineage ALL when probed with IgHJ. This is in agreement with other published studies (Korsmeyer et al, 1983; Pelicci et al, 1985). Seventeen per cent of patients with AML had rearrangements of TCRδ which was considerably less than in the series reported by Dyer where 18 of 31 patients (58%) entered into the MRC AML 9 study demonstrated rearranged bands (Dyer et al, 1988), but more in keeping with the incidence of 10% found by a Japanese group (Asou et al, 1989). Of the patients who demonstrated tumour marker bands with this probe, one had a very immature phenotype with mixed lineage markers, TdT positivity and rearrangement of IgHJ, but the other 2 patients were TdT negative and of the FAB subtypes M_2 and M_4.

5.5.2. Lymphoma

All our patients with high and low grade B cell NHL had

rearrangements in IgHJ confirming the usefulness of Ig gene rearrangements as a clonal marker(Aisenberg et al, 1987; Henni et al, 1988). One patient with anaplastic T-NHL who did not demonstate TCRβ rearrangements had an undifferentiated tumour which expressed the Ki 1 (CD30) antigen. These malignancies have shown TCRβ rearrangements in 10 of 14 reported patients, IgHJ rearrangements in 3 of 7 reported patients and germline configuration in 4 of 14 reported patients (Griesser et al, 1986, Henni et al, 1988). The method used in the present study had a sensitivity of 4% and may have failed to detect the low numbers of malignant cells. In patients with HD no useful tumour markers using gene rearrangement studies were found. The TCRδ probe was used extensively as a recent study had suggested a 28% incidence of rearrangements (Tkachuk et al, 1988), however this was not confirmed in the present study. The lack of tumour markers in HD may be due to the low number of Reed-Sternberg (R-S) cells present in the nodes and it is of interest that in studies on samples specifically enriched for these cells or on selected nodes with a high proportion of R-S cells, IgHJ rearrangements were occasionally seen (O'Connor et al, 1987; Sundeen et al, 1987).

5.6. Gene rearrangements in presentation blood from patients with NHL

As gene rearrangements were found in the vast majority of NHL, it was felt that this technique would be a valid one to employ in assessing peripheral blood involvement with lymphoma cells. These have been demonstrated in the blood by morphological assessment (Garrett et al, 1979), clonal excess (Ault, 1979; Smith et al, 1984) with a sensitivity of 5 - 10% (Berliner et al, 1986), and by DNA hybridisation (Hu et al, 1985), which appeared to be the most sensitive method (Lindh et al, 1989).

In this study gene rearrangements were found in the peripheral blood in 19.4% of patients with NHL, more being found in those with advanced stage of disease (21%) or with low grade histology (25%). Similar findings have been reported by Horning in a series of 104 patients with an overall involvement of 26% (34% in low grades, 16% high grades, 33% stage III and IV) (Horning et al, 1990), and a slightly higher percentage reported by Brada with 38% involved overall, 46% of low grades, 31% of high grades and 54% of stage III and IV) (Brada et al, 1987). His series, however, included 18 relapsed patients who tend to have a higher incidence of blood involvement (Horning et al, 1990). The finding reported here of blood gene rearrangements in 40% of those with marrow involvement and 11.5% without histological evidence of marrow disease is also lower than that found by Brada

(70% and 30% respectively), but in relatively similar proportions.

This study also demonstrated the value of gene rearrangement studies in detecting tumour involvement as circulating lymphoma cells were found microscopically in only 29% of patients with positive gene rearrangements in their blood (27% by Horning and 37% by Brada) and 33% of patients with positive bone marrows. However, the significance of peripheral blood gene rearrangements is not clear, as this finding at diagnosis was not shown to correlate with a poor prognosis (Horning et al, 1990). Moreover in the same study, in patients with either high grade or low grade disease in clinical remission and off treatment, rearrangements were documented in the blood in 10% (15 patients) of which only 1 had relapsed after a median follow up of 24 months. This finding may be significant for PBSC harvesting especially in steady state, although no studies have documented the presence of gene rearrangements in blood whilst undergoing chemotherapy. In the present study, no gene rearrangements were found in the PBSC harvests from patients with lymphoma who were leucapheresed after chemotherapy.

5.7.Tumour contamination of peripheral blood stem cell harvests

The absence of gene rearrangements in tumour cells which

could be used to assess tumour contamination in PBSCH in the 3 patients with AML and 7 patients with Hodgkin's disease was a little disappointing but not wholly unexpected. Although 17% of our AMLs had demonstrated a TCRδ rearrangement, none of these patients underwent PBSC harvesting. It may be of value to use the IgHJ probe in those who were harvested. Likewise in the patients with HD, IgHJ and TCRδ probes were unhelpful, and TCRβ was negative in the one patient studied, although further work with this probe may have yielded a marker in other patients.

The lack of rearranged bands in the PBCSH from 11 patients who had tumour markers may seem encouraging. At the time of harvest one patient with ALL (BM) and one with low grade NHL (LW) were not in morphological marrow remission but 2 patients with high grade disease (RB and MP) appeared to be so. However, in view of the insensitivity of gene rearrangement studies, if the presence of malignant cells cannot be detected below even 1 in 100 normals cells this would still represent a considerable tumour burden (5.6×10^7 cells) contaminating the harvests which in this study contained a mean of 5.6×10^9 MNC.

The insensitivity and complexity of Southern blotting limits its application to the detection of residual disease. The application of new techniques such as the PCR, allows the amplification of specific sequences of

DNA and can detect as few as $1/10^5$ to $1/10^6$ malignant cells. (d'Auriol et al, 1989; Jonsson et al, 1990). PCR amplification of TCRδ and gamma sequences has been successfully applied in ALL (Hasen-Hagge et al, 1989; d'Auriol et al, 1989), as has amplification of chromosomal translocations, such as t(14;18), in NHL and more recently in HD (Stetler Stevenson et al, 1990). The detection of residual disease by PCR techniques in peripheral blood stem cell harvests is being investigated by my collegues. A further advance in the detection of HD has been reported by the establishment of an in vitro culture system for the detection of R-S like cells, which have been found in bone marrow harvests and in 36% of peripheral blood stem cell harvests (Weisenburger et al, 1990). In neuroblastoma, a non-haematological solid tumour, a highly specific and sensitive immunocytologic assay for neuroblastoma cells with a sensitivity of 1 in 5,000 has demonstrated contamination in 14% PBSCH (Moss et al, 1990).

Although minimal residual disease will be detected using these very sensitive techniques, the clinical relevance of low levels of contamination is uncertain and will only be shown by the appropriate clinical and laboratory trials.

5.8. Concluding remarks

PBSC represent a viable alternative to bone marrow stem

cells for autologous transplantation. Standard chemotherapy regimes used in the treatment of the majority of patients with acute leukaemias, high grade non Hodgkin's lymphoma and in two thirds of those with Hodgkin's disease can induce a rise in CFU-GM at predictable times. Bone marrow involvement and previous chemotherapy reduce the peak levels of CFU-GM found in patients with lymphoma. These progenitors can be harvested in sufficient numbers for engraftment by well-timed leukapheresis and they can be cryopreserved without significant loss of viability. Using gene rearrangement studies the harvests appeared clear of tumour contamination.

The role of PBSCT in relation to BMT with regard to disease control is still uncertain and will only be clarified as clinical experience grows. The ability to collect stem cells without the need for a healthy bone marrow allows intensive therapy to be offered to more patients. The advantages of more rapid haematopoietic reconstitution from stem cells collected after chemotherapy induced mobilisation may reduce the risks of aplasia associated with transplantation. The use of combined bone marrow and PBSC transplantation to hasten haematopoietic recovery is an attractive and successful option (Bell et al, 1987a; Lopez et al, 1991) which may serve to reduce the concern over sufficient dose of PBSC to avoid secondary pancytopenias, particularly thrombocytopenia.

Recent developments such as the use of haematopoietic growth factors to mobilise PBSC, the ability to monitor rapidly the number of stem cells present in the blood by flow cytometric immunophenotyping and improvements in apheresis techniques will serve to make collection of progenitors more efficient and ultimately the outcome of PBSCT safer.

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APPENDIX 1

Presentations and Publications

Craig, J.I.O., Parker, A.C. and Anthony, R.S. (1990). The effects of various chemotherapy regimes on the levels of peripheral blood stem cells in patients with lymphoma. Bone Marrow Transplantation, **5 (suppl 1)**, 30-31.

Langlands, K,. Craig, J.I.O., Parker, A.C. and Anthony, R.S. (1990). Molecular determination of minimal residual disease in peripheral blood stem cell harvests. Bone Marrow Transplantation, **5 (suppl 1)**, 64-65.

Craig, J.I.O., Parker, A.C., Lumley, S.P., Anderson, A., Holgate, S.R. and Anthony, R.S. (1990). The collection of peripheral blood stem cells from lymphoma patients. Bone Marrow Transplantation, **5 suppl 1**, 72. (Poster).

The above were presented to the First International Symposium on Peripheral Blood Stem Cells, Mulhouse, France, Autumn, 1989.

Craig, J.I.O., Smith, S.M., Parker, A.C. and Anthony, R.S. (1990). The response of circulating stem cells to various chemotherapy regimes in patients with lymphoma. British Journal of Haematology, **74 (suppl 1)**, 15.

Presentation as finalist in the Van Der Molen prize for leukaemia research at the British Society for Haematology, Cambridge, 1990.

Langlands, K., Craig, J.I.O., Parker, A.C. and Anthony, R.S. (1990) Molecular determination of tumour contamination in peripheral blood stem cell harvests. Experimental Hematology, **18**, 680 (Poster).

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Auger, M.J., Ross, J.A., Ross, F.M., Ford, A., Russell, L., Craig, J.I.O. and Mackie, M.J. (1991). CD7 positive acute myeloblastic leukaemia. British Journal of Haematology, **77 (suppl 1)**, 25.
Presented at the British Society of Haematology, Glasgow, 1991.

Craig, J.I.O., Parker, A.C. and Anthony, R.S. Circulating progenitor cells in myelofibrosis: the effect of recombinant α2b interferon in vivo and in vitro. (1991). British Journal of Haematology, **78**, 155-160.

THE END